## *Search in UniProtKB/Swiss-Prot: There are matches to 2 out of 289473 entries*

URE1_HELFE (**Q08716**)
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URE23_HELFE (**Q08715**)
Urease subunit alpha (EC 3.5.1.5) (Urea amidohydrolase subunit alpha). {GENE: Name=ureA} - Helicobacter felis

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## *Search in UniProtKB/TrEMBL: There are matches to 6 out of 5035267 entries*

A6MEX5_HELFE
Urease (EC 3.5.1.5) {GENE:Name=ureB} - Helicobacter felis
A6MEX9_HELFE
Urease accessory protein ureG {GENE:Name=ureG} - Helicobacter felis
Q8KIZ7_HELFE
Urease UreA (Fragment) {GENE:Name=ureA} - Helicobacter felis
Q8KT24_HELFE
Urease (EC 3.5.1.5) (Fragment) {GENE:Name=ureB} - Helicobacter felis
Q9R5F5_HELFE
Urease small subunit (Fragment) - Helicobacter felis
Q9RGP5_HELFE
Urease (Fragment) {GENE:Name=ureB} - Helicobacter felis

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# UreA2B2: a second urease system in the gastric pathogen *Helicobacter felis*

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Received 13 September 2006; revised 3 November 2006; accepted 27 November 2006. First published online 12 February 2007.

DOI:10.1111/j.1574-695X.2007.00212.x

Editor: Alex van Belkum

**Keywords**
*Helicobacter*; urease; transcription; gene cluster.

## Abstract

Urease activity is vital for gastric colonization by *Helicobacter* species, such as the animal pathogen *Helicobacter felis*. Here it is demonstrated that *H. felis* expresses two independent, and distinct urease systems. *H. felis* isolate CS1 expressed two proteins of 67 and 70 kDa reacting with antibodies to *H. pylori* urease. The 67-kDa protein was identified as the UreB urease subunit, whereas the N-terminal amino acid sequence of the 70-kDa protein displayed 58% identity with the UreB protein and was tentatively named UreB2. The gene encoding the UreB2 protein was identified and located in a gene cluster named *ureA2B2*. Inactivation of *ureB* led to complete absence of urease activity, whereas inactivation of *ureB2* resulted in decreased urease activity. Although the exact function of the UreA2B2 system is still unknown, it is conceivable that UreA2B2 may contribute to pathogenesis of *H. felis* infection through a yet unknown mechanism.

## Introduction

Members of the genus *Helicobacter* are gram-negative microaerophilic bacteria that chronically colonize the gastrointestinal and hepatobiliary tract of both human and animals. Colonization with *Helicobacter* species results in chronic inflammation, which can progress into ulceration, atrophy, metaplasia and gastrointestinal and hepatic malignancies (reviewed in Solnick & Schauer, 2001; Kusters *et al.*, 2006). *Helicobacter felis* is a gastric pathogen of cats and dogs (Lee *et al.*, 1988; Paster *et al.*, 1991; Eaton *et al.*, 1996), but it can also infect mice, where it is used as animal model for *H. pylori* infection in humans (Lee *et al.*, 1990; Enno *et al.*, 1995; Nedrud, 1999).

Urease is a major virulence factor of gastric *Helicobacter* species. This enzyme hydrolyses urea into bicarbonate and ammonia, a reaction thought to be crucial in buffering the periplasm and/or cytoplasm of gastric *Helicobacter* species when colonizing their acidic gastric habitat (Stingl *et al.*, 2002; Marcus *et al.*, 2005). Ammonia is probably also used as a nitrogen source (Williams *et al.*, 1996), and is thought to assist in damaging the mucosal barrier, thereby releasing nutrients for the bacterium and maintaining the inflammation process (Smoot *et al.*, 1990). The vital role of urease in colonization by gastric *Helicobacter* species is exemplified by the finding that urease-negative mutants are unable to colonize the stomach in different animal models (Eaton & Krakowka, 1994; Tsuda *et al.*, 1994; Andrutis *et al.*, 1995).

The *Helicobacter* urease enzyme consists of two subunit proteins, UreA (27 kDa) and UreB (62 kDa) (Dunn *et al.*, 1990; Hu & Mobley, 1990). In *H. pylori* the *ureA* and *ureB* genes are organized in an operon transcribed from a promoter upstream of the *ureA* gene (Labigne *et al.*, 1991). Downstream of the *ureAB* genes, the urease operon consists of genes encoding the UreEFGH accessory proteins, which are required for the activation of the urease enzyme (Cussac *et al.*, 1992), and the UreI protein, which is an inner membrane protein that functions as a $H^+$ gated urea channel. This channel allows access of the urea substrate from the surrounding environment to the cytoplasm of the bacterium (Weeks *et al.*, 2000).

Although *H. felis* has been extensively used for studies on gastritis and vaccination against *Helicobacter* (reviewed in

Solnick & Schauer, 2001; Kusters *et al.*, 2006), surprisingly little is known about the function and regulation of its virulence factors, such as urease. The *ureAB* gene cluster of *H. felis* was previously identified and showed high homology to the *H. pylori* urease system (Ferrero & Labigne, 1993), but further information is not available. Therefore we have set out to characterize the urease system of *H. felis*.

## Materials and methods

### Bacterial isolates and growth conditions

*Helicobacter felis* isolate CS1 (CCUG28539) was grown on Jalava plates [Brain Heart Infusion (BHI) plates (Oxoid, The Netherlands) supplemented with 1 g $L^{-1}$ yeast extract (Becton Dickinson, The Netherlands), 20 g $L^{-1}$ Bacto agar (Becton Dickinson), 7% defibrinated horse blood (BioTRADING, The Netherlands), amphotericin B (5 mg $L^{-1}$) and Skirrow antibiotic supplement (Oxoid)]. *Helicobacter felis* is highly motile, and requires slightly moist plates for growth. Consequently, *H. felis* grows as a lawn (Gottwein *et al.*, 2001). Therefore, mutants were isolated by repeated subculturing limiting dilution series on chloramphenicol-containing agar plates. For broth cultures *H. felis* strains were grown in BHI broth supplemented with 10% newborn calf serum (Invitrogen, The Netherlands). Bacteria were incubated at 37 °C in a microaerophilic atmosphere of 5% $O_2$, 10% $CO_2$ and 85% $N_2$. *Escherichia coli* strain DH5α was cultured in Luria–bertani (LB) media. When appropriate, chloramphenicol was added to growth media to a final concentration of 10 mg $L^{-1}$.

### General genetic manipulations

Plasmid DNA was isolated and purified with the Wizard® Plus SV minipreps DNA purification system (Promega, The Netherlands). All DNA modifying enzymes were purchased from Promega, and were used according to manufacturers descriptions. PCR amplification reactions were performed in an automated thermal cycler (I-Cycler; Bio-Rad) using Taq polymerase (Promega).

### Construction of *Helicobacter felis* urease mutants

Part of the *ureAB* and *ureA2B2* gene clusters of *H. felis* isolate CS1 was amplified using the primer combinations HfUreABF/HfUreABR and HfUreA2B2F/HfUreA2B2R, respectively (Table 1). The resulting amplicons were subsequently cloned in pGEM-$T_{easy}$ vector (Promega) and transformed into *E. coli* strain DH5α using standard techniques (Sambrook *et al.*, 1989). A single random colony was selected and correct insertion was confirmed by sequence analysis of the insert. Plasmid pAV35 was the source of the chloramphenicol resistance cassette ($Cm^R$-cassette) (van Vliet *et al.*, 1998). After cutting with *Cfo*I, the $Cm^R$ cassette was introduced into the cloned *ureAB* and *ureA2B2* fragments cut with *BsmI*, resulting in constructs pUreabCm and pUrea2b2Cm. The constructs were then transformed to *H. felis* isolate CS1 by electroporation using a minor modification of a previously described protocol (Josenhans *et al.*, 1999), as recovery after electroporation was performed for 16 h on Jalava plates. Subsequently, bacteria were transferred to Jalava plates containing 10 or 20 mg $L^{-1}$ chloramphenicol and were incubated for 8–10 days. Mutant strains resistant to chloramphenicol were selected by repeated subculturing limiting dilution series on chloramphenicol-containing agar plates. Correct replacement of the wild-type urease genes by the interrupted copies was confirmed by PCR using combinations of the primers HfUreABF2, HfUreABR2, HfUreA2B2F2, HfUreA2B2R2, CatoutF, and CatOutR (Table 1).

**Table 1.** Oligonucleotide primers used in this study

| Primer | Sequence (5′ → 3′) |
|---|---|
| HfUreABF | CGGAAAGAAGCCATTAGC |
| HfUreABR | CTTCAGCCGCGATAGTTTG |
| HfUreABF2 | ACTATCCACACTCCGGTAGA |
| HfUreABR2 | TTGTCTGCTGTCTGCCAAGT |
| HfUreA2B2F | TGATCCTGTGTTGTGGGTGGTA |
| HfUreA2B2R | CACGATCGCACACGCAAGAC |
| HfUreA2B2R2 | GAGGCGATGGGTTTCAATAC |
| HfUreA2B2F2 | GGCGAAGTGGCTAGAA |
| CatoutF | TAGTGGTCGAAATTACTCTTTTCGTG |
| CatoutR | CCCTTATCGATTCAAGTGCATCATG |
| HfurelprobeF | ATTGTATGTTGCGGTCGTGCTG |
| HfurelprobeR* | ctaatacgactcactatagggagaGGCCGCTGGGATAGTGTTG |

*Primer contains a 5′-extension consisting of the T7 promoter sequence (lowercase letters) for the creation of an antisense RNA probe.

### Urease assay

The enzymatic activity of urease was determined by measuring ammonia production from hydrolysis of urea, using the Berthelot reaction (van Vliet *et al.*, 2001). The concentration of ammonia in the samples was calculated from a standard $NH_4Cl$ concentration curve. Enzyme activity was expressed in micromoles of urea hydrolyzed per minute per milligram of protein.

### Protein analysis

Broth cultures were centrifuged for 10 min at 13 000 *g* and resuspended in phosphate-buffered saline to a final $OD_{600\,nm}$ of 10. Bacteria were diluted 1:10 and subsequently lysed by sonication for 15 s with an MSE Soniprep 150 set at amplitude 6. Protein concentrations were determined with the BCA protein Assay kit (Pierce, The Netherlands). Total

protein was separated by SDS-PAGE, followed by staining with Coomassie brilliant blue or immunoblotting. Expression of urease subunits was monitored on immunoblots using 1:1000 diluted *H. pylori* urease-specific antiserum (Phadnis *et al.*, 1996). The N-terminal amino acid sequence of the 67- and 70-kDa proteins was determined by Edman degradation.

### RNA analysis

Total RNA was isolated from *c.* $5 \times 10^8$ *H. felis* cells using TriZOL (Invitrogen). RNA was visualized by methylene blue staining, and RNA concentrations were normalized based on 16S and 23S rRNA gene band intensities (van Vliet *et al.*, 2001). An internal fragment of the *ureI* gene was amplified with primers HfureIprobeF and HfureIprobeR (Table 1). The PCR fragment contained a T7 promoter sequence and was used for the production of an antisense RNA probe labeled with digoxigenin by transcription with T7 RNA polymerase (Roche Diagnostics, The Netherlands). Northern hybridization and stringency washes were performed at 68 °C, and a bound probe was visualized with the DIG-Detection Kit (Roche Diagnostics) and chemiluminescent substrate CPD-Star (Amersham Pharmacia, Sweden) (van Vliet *et al.*, 2001). The sizes of the hybridizing RNA were calculated from comparison with a digoxigenin-labeled RNA size marker (RNA Marker I; Roche Diagnostics).

### Accession numbers

The sequences of the *H. felis* CS1 *ureABIEFGH* and *ureA2B2* urease gene clusters have been deposited in the GenBank sequence database under accession numbers DQ865138 and DQ865139, respectively.

### Phylogenetic analysis

Protein sequences of the large urease subunits of *E. coli* O157:H7 EDL933 (UreC, accession number AAG55290), *Campylobacter lari* CF89-12 (UreB, accession number BAD89502) *Helicobacter hepaticus* ATCC51449 (UreB, accession number AAP77005) and *H. pylori* 26695 (UreB, accession number AAD07143) were retrieved from the GenBank database. Multiple sequence alignments were made using the CLUSTALW program (Thompson *et al.*, 1994), and a phylogenetic tree was constructed using the PROTDIST and NEIGHBOR programs of the PHYLIP 3.66 software package (Felsenstein, 1989).

### Statistical analysis

Statistical analyses were performed using the Wilcoxon Signed Ranks test. Two-sided $P$ values $< 0.05$ were considered significant.

## Results

### *Helicobacter felis* expresses multiple proteins reacting with urease antibodies

To monitor urease expression, lysates from *H. felis* isolate CS1 and *H. pylori* strain 26695 were analyzed by immunoblotting using an antiserum raised to purified *H. pylori* urease enzyme (Phadnis *et al.*, 1996). In the *H. pylori* lane, two immunoreactive bands of *c.* 27 and 62 kDa were detected, which correspond to the UreA and UreB proteins (Fig. 1). The urease antiserum showed only mild binding in the *H. felis* CS1 lane in the area corresponding to the UreA protein, but displayed strong antibody-binding towards two separate proteins of 67 and 70 kDa, respectively (Fig. 1).

The N-terminal amino acid sequences of the 67- and 70-kDa *H. felis* proteins were subsequently determined. The N-terminal amino acid sequence of the 67-kDa protein was identical (20/20 residues) to that of the *H. felis* UreB protein (Ferrero & Labigne, 1993), and was therefore designated UreB. The N-terminal amino acid sequence of the 70-kDa protein was different from the 67-kDa protein, but displayed homology to the UreB protein (Ferrero & Labigne, 1993), with 11/19 residues identical, and was tentatively named UreB2 (Fig. 1).

### *Helicobacter felis* contains two independent gene clusters encoding putative urease systems

The regions containing the *ureB* and *ureB2* genes were subsequently cloned from the *H. felis* isolate CS1 by PCR using primers based on conserved regions in the protein sequences. As predicted, the *H. felis ureB* gene was preceded

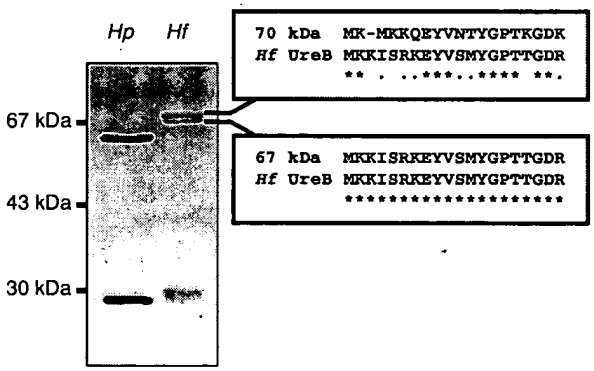


**Fig. 1.** *Helicobacter felis* expresses two UreB-like proteins. Immunoblot incubated with polyclonal antibodies raised to *H. pylori* urease. Lane 1, *Helicobacter pylori* (*Hp*); lane 2, *H. felis* CS1 (*Hf*). The 67 and 70 kDa bands reacting with the anti-*H. pylori* urease antibodies are indicated on the right, relevant marker sizes on the left. The N-terminal amino acid sequences of the 67- and 70-kDa proteins are given, aligned with the *H. felis* UreB sequence. Asterisks indicate identical residues, dots indicate conservative substitutions.

by the *ureA* gene (Ferrero & Labigne, 1993), and contained an operon downstream containing orthologs of the *H. pylori ureIEFGH* genes (Fig. 2). Upstream of *ureB2* a gene (*ureA2*) was present putatively encoding a protein homologous to the *H. felis* UreA protein. The *ureA2* and *ureB2* genes were present in five other independent *H. felis* isolates, as tested by PCR using a primer located in the *ureA2* and a primer located in the *ureB2* gene (data not shown). We have subsequently tried to identify the genes downstream of *ureB2*, but repeated attempts with either inverse PCR and degenerate primers based on *ureI* and *ureE* sequences were unsuccessful (data not shown), suggesting that accessory genes are absent downstream of *ureB2* (Fig. 2), although further investigation is required to confirm this.

Analysis of the protein sequences encoded by the *ureAB* and *ureA2B2* operons showed that the UreA and UreA2 proteins display 46% sequence identity, whereas the UreB and UreB2 proteins display 73% sequence identity. When compared to other *Helicobacter* species the UreA2 protein displays 48–54% sequence identity and the UreB2 protein display 68–74% sequence identity. The UreB and UreB2 proteins both contain the predicted active site of urease (Hulo *et al.*, 2004) as well as the predicted nickel binding site (Mobley *et al.*, 1995).

## Mutation of the *ureB2* gene affects overall urease activity of *Helicobacter felis*

To determine whether the UreA2B2 system encodes a functional urease enzyme, we inactivated the *ureB* and *ureB2* genes of *H. felis* CS1 by insertional mutagenesis (Fig. 2). Unfortunately, despite repeated attempts the kanamycin resistance cassette that has been frequently used to generate mutants in *H. pylori* did not generate any kanamycin resistant colonies in *H. felis* (data not shown). As a consequence, only the chloramphenicol resistance cassette could be used for the generation of mutants in *H. felis*, and thus it was not possible to construct a double mutant with both genes, *ureB* and *ureB2*, inactivated.

Urease activity of the *H. felis* isolate CS1 and isogenic mutants was compared to determine the relative contribution of the UreAB and UreA2B2 systems in *H. felis* urease activity (Fig. 3). Mutation of the *ureB* gene resulted in a complete absence of urease activity, suggesting that the UreA2B2 urease is inactive under *in vitro* growth conditions. However, mutation of the *ureB2* gene also resulted in a modest but significant decrease in urease activity (Fig. 3), suggesting that the UreA2B2 urease does contribute to overall urease activity of *H. felis*.

## The urease-negative phenotype of the *ureB* mutant is not due to effects on transcription of urease accessory genes

To determine whether the discrepancy between the effects of the inactivation of *ureB* and *ureB2* on urease activity of these mutants was caused by polar/downstream effects of insertion of the chloramphenicol cassette in the *ureB* gene, the effect on the transcription of the downstream *ureIEFGH*

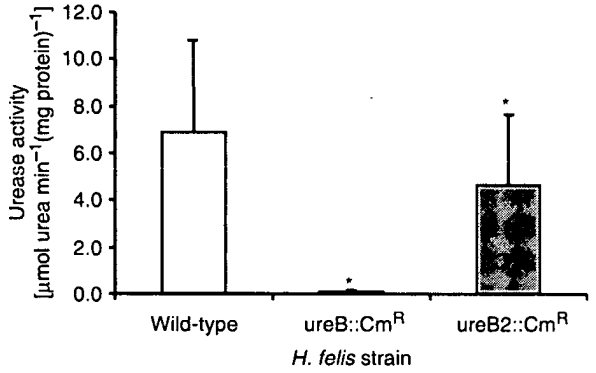


**Fig. 3.** Effect of *ureB* and *ureB2* inactivation on *Helicobacter felis* urease activity. Urease activity measurements of wild-type *H. felis* CS1 (white bar), *ureB* mutant (black bar) and *ureB2* mutant (grey bar), grown in broth culture. Results show the average of 5-7 independent urease activity measurements. The error bars denote SDs, the asterisks indicate a significant difference in urease activity of the mutants when compared to the wild-type strain ($P < 0.05$, Wilcoxons Signed Ranks test).

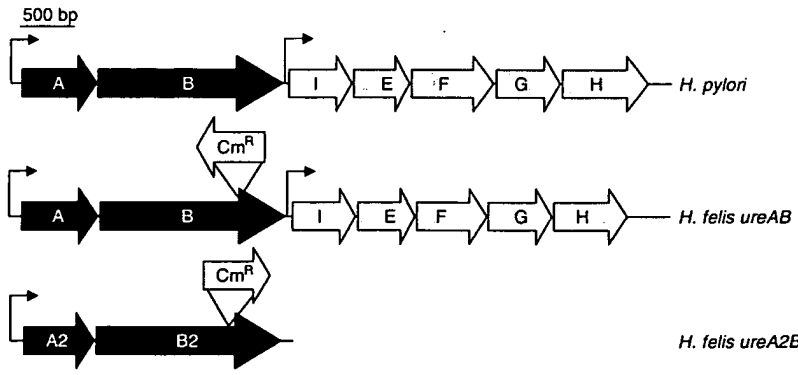


**Fig. 2.** Genetic organization of the *Helicobacter pylori* and two *H. felis* urease gene clusters and the orientation and insertion site of the chloramphenicol cassette (Cm$^R$) in the *H. felis* mutants. Small arrow symbols indicate the putative promoters upstream of *ureA*, *ureI* and *ureA2* predicted to drive transcription of the urease operons.

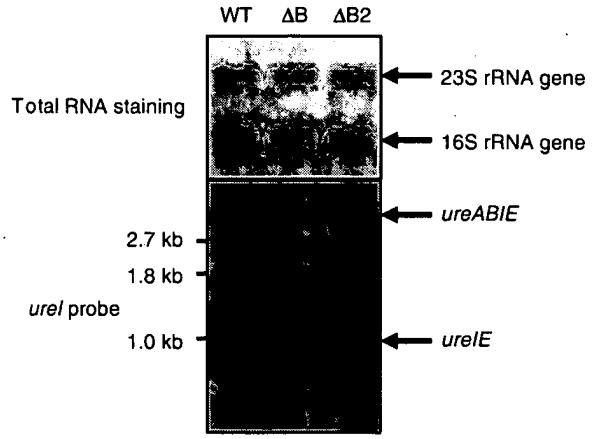


**Fig. 4.** Absence of urease activity in the *Helicobacter felis ureB* mutant is not caused by a polar effect on transcription of the downstream urease accessory genes. The figure shows a Northern hybridization with a *ureI* probe. WT, *H. felis* CS1 (wild-type strain);ΔB, *H. felis* CS1 *ureB* mutant (*ureB*::$Cm^R$);ΔB2, *H. felis* CS1 *ureB2* mutant (*ureB2*::$Cm^R$). Staining of transferred RNA by methylene blue is included for comparison of RNA amounts (top panel). The position of the predicted *ureABIE* and *ureIE* transcripts is indicated on the right-hand side, whereas the probe used and relevant marker sizes are included on the left-hand side.

operon was determined by Northern hybridization. Based on the situation in *H. pylori*, it was anticipated that a *ureI*-specific probe should allow us to evaluate the transcription originating from the predicted *ureA* and *ureI* promoters in *H. felis* (Akada *et al.*, 2000; van Vliet *et al.*, 2001). In the wild-type *H. felis* isolate, the *ureI* probe reacted with two mRNA species of *c.* 1 and 3 kb, predicted to represent the *ureABIE* and *ureIE* mRNAs, respectively (Akada *et al.*, 2000; van Vliet *et al.*, 2001). In the *ureB* mutant the *ureABIE* mRNA is missing, but the *ureIE* mRNA is still present, albeit in lower amounts (Fig. 4). In the *ureB2* mutant both the *ureABIE* and the *ureIE* mRNAs are still present, and therefore the absence of UreA2B2-associated urease-activity in the *ureB* mutant is probably not caused by lack of transcription of the *ureIEFGH* operon including the accessory genes.

## Discussion

The urease enzyme is important in the pathogenesis of *Helicobacter* infection, as shown by the inability of urease-negative mutants of different *Helicobacter* species to colonize their respective hosts (Eaton & Krakowka, 1994; Tsuda *et al.*, 1994; Andrutis *et al.*, 1995) While the urease system of *H. pylori* has been extensively studied, relatively little is known about the urease systems of other *Helicobacter* species.

Part of the *H. felis* urease gene cluster was previously identified in part, as the sequence of the *ureA* and *ureB* genes was published (Ferrero & Labigne, 1993). When we tested whether the urease of *H. felis* shared cross-reactive epitopes with *H. pylori* urease, we noticed that antiserum to *H. pylori* urease recognized two proteins bands of 67 and 70 kDa in *H. felis* (Fig. 1). The identity of both proteins was established by N-terminal amino acid sequencing, and the 67 kDa band was identical to the UreB protein, and the 70 kDa band contained a different N-terminal amino acid sequence which shared homology with the *H. felis* UreB protein (Fig. 1) (Ferrero & Labigne, 1993). Therefore we set out to characterize the gene encoding this putative second urease subunit, and we have also extended the characterization of the gene cluster downstream of the *ureB* gene. The *ureB* and *ureB2* genes are located in two separate gene clusters, one cluster containing both structural and accessory genes (*ureAB* and *ureIEFGH*), and one cluster only containing the *ureA2B2* genes (Fig. 2). Both the sequence and organization of the complete urease gene cluster is similar to that of *H. pylori* (Mobley *et al.*, 1995) and other *Helicobacter* species (Zhu *et al.*, 2002). Also, the *ureA2B2* gene cluster was present in all five additionally tested *H. felis* isolates (strains CS7, 2301, 144, 1538 and 390) (data not shown).

The UreA2 and UreB2 proteins are 46% and 73% identical to the UreA and UreB proteins, respectively, and are phylogenetically most closely related to the *Helicobacter* UreA and UreB proteins (Fig. 5). In view of the conservation of the active site and nickel-binding site present in the UreB2 protein, this makes it likely that the UreA2B2 complex represents a urease enzyme. However, the data obtained using insertional mutagenesis of the *ureB* and *ureB2* genes contradict each other. In view of the absence of urease activity in the *ureB* mutant, it can be concluded that UreA2B2 does not contribute to total urease activity, whereas the reduction in urease activity in the *ureB2* mutant suggests differently (Fig. 3). The phenotype of the *ureB* mutant is probably not caused by lack of enzyme activation via absence of urease accessory proteins, as the presence of the *ureIE* mRNA suggests that transcription from the *ureI* promoter still occurs (Fig. 4). Although unlikely, there may

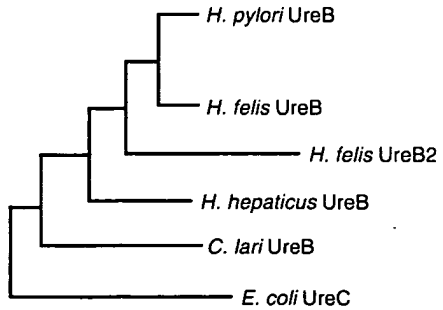


**Fig. 5.** Phylogenetic tree displaying the evolutionary relationship between the *H. felis* UreB2 protein and other urease large subunits (UreB/UreC). The tree was constructed from a distance matrix, calculated from a multiple sequence alignment of the respective sequences.

also be polar effects of the introduction of the chloramphenicol resistance cassette in the *ureB* and *ureB2* genes. This issue can however only be addressed by genetic complementation, which is currently not feasible in *H. felis*.

The presence of two urease gene clusters is not unique in bacteria, as it has been described in the genome sequences of *Brucella suis* and enterohaemorrhagic *E. coli* (Perna *et al.*, 2001; Paulsen *et al.*, 2002). However, in both *B. suis* and enterohaemorrhagic *E. coli* the two urease gene clusters include accessory genes, while our data thus far indicate that in *H. felis* only a single set of accessory genes is present, directly downstream of the *ureAB* genes (Fig. 2). The UreA2 and UreB2 proteins cluster together with the other *Helicobacter* UreA and UreB proteins (Fig. 5), and the *H. felis* UreA and UreA2 proteins, are both fusions of the ancestral bacterial beta and gamma subunits (Mobley *et al.*, 1995; Burne & Chen, 2000), suggesting that the *ureA2B2* gene cluster has not been recently acquired by horizontal gene transfer.

The function and regulation of the UreA2B2 system is currently unknown. It is tempting to speculate that the presence of two separate urease systems may allow differential expression of urease systems depending on the environmental conditions. The presence of the second urease should also be taken into account with both infection and vaccination studies using the *H. felis* mouse model, as cross-neutralization or cross-protection between both antigens is likely, but may not result in full protection. Further investigations are needed to determine the possible role of the second urease in gastrointestinal and hepatic malignancies.

## Acknowledgements

We thank K. Jalava for donating *H. felis* strains CS1 and 2301, R. van der Schors for N-terminal amino acid sequencing, S. Kuijper and P. van Nuenen for technical assistance, and A. Bart, G. Cattoli and C.M.J.E. Vandenbroucke-Grauls for helpful suggestions and discussions.

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Vet Pathol 36:237–248 (1999)

# *Helicobacter felis* Infection in Dogs: Effect on Gastric Structure and Function

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**Abstract.** The relationship of *Helicobacter felis*, an organism that is observed in the stomachs of dogs, to gastric disease in dogs is unclear. The objective of this study was to determine if *Helicobacter felis* infection alters gastric morphology and gastric secretory function in dogs. Five specific-pathogen-free (SPF), *Helicobacter*-free Beagle dogs were examined before and for 26 weeks after inoculation with *H. felis* (ATCC 49179). Three SPF uninfected dogs served as controls. All five dogs became colonized by *H. felis* as determined by urease activity, histopathology, polymerase chain reaction, and transmission electron microscopic examination of serial gastric biopsies. The degree of colonization ranged from <1 organism/400× field to >10 organisms/400× field. The fundus, body, and cardia were most heavily colonized. Evaluation of gastric biopsies showed mild gastric inflammation and lymphoid follicles in both infected and uninfected dogs. There was no correlation between the number of organisms observed and the degree of gastric inflammation or number of lymphoid follicles. The gastric secretory axis, assessed by fasting and meal-stimulated plasma gastrin, mucosal gastrin and somatostatin immunoreactivity, fasting gastric pH, and pentagastrin-stimulated gastric acid secretion, was similar in both infected and uninfected dogs. Fasting gastric pH was not a reliable indicator of gastric secretory function. These findings suggest that *H. felis* may not be a gastric pathogen in dogs. However, the density of colonization and limited duration of infection should be considered when interpreting these findings.

*Key words:* Dogs; gastric acid; gastrin; gastritis; *Helicobacter;* somatostatin.

The discovery of the association of *Helicobacter pylori* with gastritis, peptic ulcers, and gastric neoplasia has led to fundamental changes in the understanding of gastric disease in humans.[2,29,44,46] Investigation of the relationship of gastric disease with *Helicobacter* infection in other species has resulted in the discovery of *H. mustelae* in ferrets with gastritis and peptic ulcers, *H. acinonyx* in cheetahs with severe gastritis, and *H. heilmannii* in pigs with gastric ulcers.[7,15,42] The presence of gastric *Helicobacter*-like organisms (HLO) in the stomachs of dogs has been known for many years,[1] but the relationship of these organisms to gastric disease is unresolved, with inflammation accompanying infection in some but not all infected dogs.[5,19,24–26,48,49] Infection with HLO is highly prevalent in dogs; it is seen in 61–80% of dogs presented for the investigation of vomiting,[19,26,49] 67–86% of clinically healthy pet dogs,[5,49] and almost 100% of laboratory Beagles and shelter dogs.[5,6,25] The gastric HLO in dogs are morphologically indistinguishable by light microcopy, where they are seen as large spirals 5–12 μm long, but have been classified into several *Helicobacter* species on the basis of 16S ribosomal RNA sequencing, DNA hybridization, and electron microscopic appearance.[5,21,25,32] To date, *H. felis, H. bizzozeronii, H. salomonis, H. bilis,* and *Flexispira rappini* have been identified.[5,21,27,30,32] The presence of multiple species of organisms complicates the investigation of pathogenicity, but experiments to determine the pathogenicity of *H. felis* and *H. pylori* have demonstrated gastritis and humoral immune response in gnotobiotic dogs after infection.[31,43] However, clinical signs were absent in those dogs. Those studies also report an increase in fasting gastric pH in 4 of 10 dogs after infection.[31,43] Increases in gastric pH and achlorhydria have been reported in some humans with *H. pylori* infection.[8,36,45] *H. felis* and *H. pylori* also decrease parietal cell acid secretion in vitro.[3,47] However, other studies in humans indicate that *H. pylori* infection is related to increased gastric acid secretion.[9,10,37] In people infected with *H. pylori,* increased acid secretion is associated with antral gastritis and duodenal ulceration,[9,37] whereas achlorhydria is observed shortly after infection with *H. pylori* and when the gastric fundus and body is inflamed or atrophied.[8,34,36,45] Eradication of *H. pylori* has been associated with decreased acid secretion in patients with acid hypersecretion and increased acid secretion in achlorhydric patients.[8,9,37] Decreased inhibition of gastrin release by somatostatin, with resultant hypergastrinemia and increased parietal cell mass, has

been postulated as the cause of hyperacidity and duodenal ulceration.[38,41] Inhibition or destruction of parietal cells is considered responsible for achlorhydria.[3,8,47] In contrast to the variability of gastric acid secretion that accompanies *H. pylori* infection, the magnitude of hypergastrinemia in asymptomatic individuals, those with achlorhydria, and those with duodenal ulcers is similar.[9,20] The effect of *H. felis* infection on the gastric secretory axis of dogs has not been critically examined. We report here the evaluation of gastric morphology, acid secretion, fasting and meal-stimulated serum gastrin, and gastric somatostatin and gastrin immunoreactivity after experimental infection of dogs with *H. felis*.

## Materials and Methods

### Animals

Five specific-pathogen-free (SPF), *Helicobacter*-free Beagle dogs (4 months old, two female, three male, weight [$\bar{x} \pm$ SE] = 8.2 ± 0.57 kg) from the Baker Institute for Animal Health, Cornell University, were studied before and for 26 weeks after oral inoculation with *H. felis*. Three SPF, *Helicobacter*-free dogs (4 months old, one female, two male, weight = 8.5 ± 0.37 kg) that had not been infected with *H. felis* served as uninfected controls. The presence or absence of gastric *Helicobacter* spp. was ascertained in the eight dogs prior to admission to the study by evaluating gastric biopsies for urease activity, impression smears and tissue sections for the presence of HLO, and gastric biopsies for *H. felis* or other *Helicobacter* DNA. All dogs were negative for *Helicobacter* on all tests. Dogs were acclimatized to housing for 2 weeks prior to starting the study and for 4 weeks before infection with *H. felis*. Dogs were fed a standard commercial diet and had constant access to water throughout the study. Infected dogs were housed separately from uninfected dogs. Cornell University operates under an approved Animal Welfare Assurance (A3347-01) and is fully accredited by the American Association for the Accreditation of Laboratory Animal Care. The project was approved by the Institutional Animal Care and Use Committee at Cornell University.

### Experimental design

Dogs were anesthetized 2 weeks prior to and 12 and 25 weeks after inoculation with *H. felis* for the evaluation of gastric acid secretion and gastric biopsy. Gastric biopsies were obtained endoscopically for histopathology, impression smears, urease testing, and polymerase chain reaction (PCR) analysis. Two weeks prior to the oral administration of *H. felis* and at scheduled intervals 2–24 weeks after blood was obtained for determination of gastrin. Meal-stimulated gastrin release was evaluated at 0, 6, 12, and 24 weeks. Blood samples were collected at 0, 12, and 24 weeks after infection for serology. Comprehensive gastric urease mapping, histology, electron microscopy, PCR analysis and enumeration of immunoreactive somatostatin and gastrin cells was performed for gastric biopsies obtained after euthanasia at 6 months.

### Infection with *H. felis*

*Helicobacter felis* strain ATCC 49179 was used; it was originally isolated from the gastric mucosa of an adult cat.[30] *H. felis* was grown on 60 horse serum agar biphasic slants (trypticase soy agar base [Difco Labs, Detroit, MI] with 10% horse serum incorporated and trypticase soy broth overlay) for 2 weeks prior to animal inoculation. On the day of harvest, bacteria from four slants were gently vortexed and combined into a single tube. The bacteria were checked by Gram's stain to ensure that there were few or no reversions from rod to coccoid forms. The bacterial suspension was then standardized at a turbidity of 0.5 McFarland, which would normally result in about $1.5 \times 10^8$ bacteria colony-forming units/ml; however, because *H. felis* does not produce discrete colonies on agar, we could not do the standard colony counts to determine the actual number of *H. felis* in a 0.5 McFarland standard. An innoculum of 14.5 ml was administered via a stomach tube on days 1, 3, and 5 of the experiment.

### Culture of gastric biopsies

Biopsies were transported to the laboratory in trypticase soy broth tubes (BBL Microbiological Systems, Becton Dickinson, Cockeysville, MD) on ice. Upon receipt in the laboratory, the biopsies were transferred to a ten broek tissue grinder, ground, plated directly onto trypticase soy agar with 5% sheep blood plates (BBL) and onto *Brucella* blood PRAS agar plates (Anaerobe Systems, Morgan Hill, CA), and incubated in a microaerophilic atmosphere at 36 C with added moisture for 3–7 days. Plates were checked daily for growth. Suspect colonies were subcultured to a *Brucella* blood PRAS agar plate and reincubated. Direct colony gram staining also was performed.

### Gastrin

Two weeks prior to the oral administration of *H. felis* and at 2-week intervals (2–24 weeks) after, blood was collected into ethylenediaminetetraacetic acid. All sampling periods were preceded by an overnight fast. Meal-stimulated gastrin release was evaluated in the second week prior to infection and at weeks 6, 12, and 24 after infection by serial blood sampling 0, 30, and 60 minutes after a standardized peptone meal (25% peptone [w/v]; 10 ml/kg). Blood samples were placed in ice and centrifuged at 4 C, and plasma was stored at −70 C until analysis. Plasma concentrations of gastrin were determined at the Department of Endocrinology, Ohio State University, using a radioimmunoassay (Becton Dickinson) validated for dogs.

### Measurement of acid secretion

Gastric acid secretion was evaluated in anesthetized dogs prior to and at 12 and 25 weeks after oral administration of *H. felis*. Anesthesia was induced with intravenous thiopentone sodium (15 mg/kg) and maintained with halothane and oxygen via an endotracheal tube. A gastric tube (12 fr Levin tube; Davol, Cranston, RI) was positioned endoscopically in the dependent part of the stomach. The tube position was checked by injecting and recovering 6 ml of sterile water. Gastric juice was then continuously aspirated using gentle

manual suction for 75 minutes. Basal (30 minutes) and pentagastrin (30–45, 45–60, 60–75 minutes) stimulated fractions were collected on ice. The collection, stimulation, and quantitation of gastric acid secretion were as previously described[22] except that pentagastrin (Bachem Bioscience, King of Prussia, PA) was administered as a continuous intravenous infusion of 8 μg/kg/hour. Acid secretion was determined by pH measurement and titration to pH 7.0 with 0.1 M NaOH. Maximal acid output was calculated using values from the 15-minute period with the highest acidity, and acid output was expressed as pH, milliliters/hour, millimoles HCl/milliliter, millimoles HCl/hour, milliliters/kilogram$^{0.75}$/hour, and millimoles HCl/kilogram$^{0.75}$/hour.

### Gastric biopsy

Endoscopic biopsies of the stomach were obtained at −2, 12, and 25 weeks with a pediatric endoscope and biopsy forceps. Endoscopic biopsies were procured from the pyloric antrum (incisura to pyloric sphincter), the body (greater curvature), and the cardia. Three biopsies were taken from each site for light microscopy, two from each site for urease testing, and one from each site for impression smears and PCR. Endoscopic biopsy samples for PCR analysis were pooled and frozen at −70 C pending analysis. The endoscope was thoroughly cleaned and then sterilized using an activated aldehyde solution (Metrex, Parker, CO). Biopsy forceps were sterilized in a similar fashion, and the biopsy cups were immersed in chlorox (1 : 10 in water) for 10 minutes to destroy residual DNA. At necropsy (26 weeks), two full-thickness gastric biopsies were obtained from 10 standardized sites as previously described[31] using a 6-mm skin biopsy punch. One biopsy from each site was evaluated for urease activity, and the other was used for light microscopy. Additional biopsies for PCR analysis and transmission electron microscopy were obtained adjacent to biopsy sites 1 (cardia), 5 (body), and 8 (pyloric antrum).

### Biopsy urease

Biopsy urease production was evaluated as previously described.[43] Gastric mucosal biopsies were placed in sterile tubes containing 200 μl of a solution composed of urea, sodium azide, phenol red, and phosphate-buffered saline (PBS) (pH 6.5). Biopsies were incubated for 48 hours and observed at 1, 4, 24, and 48 hours for a change in the color of the indicator medium. A change from orange-red to bright pink was considered a positive result, and the time of color change was recorded. Urease results were additionally scored as follows: negative at 48 hours = 0, positive at 1 hour = 4, positive at 4 hours = 3, positive at 24 hours = 2, positive at 48 hours = 1.

### Histopathology

Impression smears were stained with Diff-Quick and evaluated by light microscopy for the presence of HLO. Samples for histopathology were fixed in 10% buffered formalin, embedded in paraffin, and sectioned at 4–6 μm. Serial sections of each block were stained with hematoxylin and eosin (HE) and modified Steiner's stain.[18] Samples were examined in a blinded fashion by one pathologist (B. A. Valentine) and evaluated for the number of organisms, degree of inflammation, and presence of mucosal lymphoid nodules. The number of organisms was graded as follows: 0 = no organisms seen, + 1 = ≤1 organism/400× field, +2 = 1–10 organism/400× field, +3 = >10 organisms/400× field. The degree of inflammation was graded as follows: 0= minimal to no mononuclear inflammatory cells, +1 = mild increase in mononuclear inflammatory cells, +2 = moderate numbers of mononuclear inflammatory cells. The number of lymphoid follicles was evaluated in biopsies obtained at necropsy. Biopsies obtained for histopathology at necropsy were grouped according to site (cardia/fundus = biopsies 1–3, body = biopsies 4–6, pyloric antrum = biopsies 7–10).[31]

### Electron microscopy

Gastric biopsies were fixed by immersion in a solution containing 2.5% gluteraldehyhe cacodylate (0.1 M) buffered to pH 7.2. Samples were postfixed in 1% osmium tetroxide, dehydrated, infiltrated, and embedded in Epon araldite. Semithin sections cut at 0.5 μm were stained with azure blue. Thin sections, approximately 80 nm thick, were stained with uranyl acetate and lead citrate and examined at 80 kV with a Philips 201 transmission electron microscope.

### Immunocytochemistry for gastrin and somatostatin

Immunohistochemistry was performed on deparaffinized tissue from the pyloric antrum using polyclonal rabbit anti-gastrin (1 : 40) (BioGenex Labs, San Ramon, CA) and anti-somatostatin (prediluted) (Zymed Laboratories, San Francisco, CA) antibodies and streptavidin–biotin immunoperoxidase technique with diaminobenzidine as the chromagen. Nonimmune rabbit serum at 1 : 80 was used as a negative control. The number of immunoreactive cells in the pyloric antrum was quantitated by two methods. The first method involved counting all immunoreactive cells observed in biopsies from the pyloric antrum (biopsy sites 7–10). In the second method, at least 10 areas (of the muscularis mucosa, 0.245 mm in length) from four sections of each biopsy were evaluated using a dry 40× objective.[38,41] Cells were counted in the midzone of the mucosa where gastrin and somatostatin cells are located. The results were expressed as the ratio of immunoreactive gastrin to somatostatin cells.

### PCR

Gastric biopsies collected endoscopically at 0, 12, and 25 weeks and at necropsy were frozen at −70 C. DNA was extracted from biopsies as previously described.[4] PCR was performed using primers that amplify the urease B gene of *H. felis*: F-5′-ATGAAACTAACGCCTAAAGAACTAG-3′ and R-5′-GGAGAGATAAAGTGAATATGCGT-3′.[39] DNA samples (2 μl from a 1 : 10 dilution) were added to a reaction mixture containing 800 μM dNTPs (Pharmacia Biotech, San Francisco, CA), PCR buffer (Gibco BRL, Grand Island, NY), 2 mM $MgCl_2$ (Gibco BRL), 1.25 units of *Taq* DNA polymerase (Gibco BRL), 0.5 μM of each primer, and distilled water in a total volume of 50 μl. PCR samples were heated to 94 C for 2 minutes followed by 40 cycles of denaturation at 94 C for 1 minute, primer annealing at 62 C for 1 minute and extension at 72 C for 1 minute, with a final extension at 72 C for 15 minute in a Perkin Elmer model 960 thermocycler. PCR products were subjected to electro-

phoresis on an agarose gel and visualized with ethidium bromide. When visualized under an ultraviolet light, a single band at 1,150 base pairs was present when *H. felis* ATCC 49179 was used. This band was absent with DNA from *H. pylori, H. bizzozeronii, H. heilmannii, H. fenelliae, H. bilis, H. cinaedii,* and *Campylobacter jejuni.*

*Helicobacter*-specific primers C97 and C05 were used to generate 16S ribosomal RNA amplicons[16] from the three SPF uninfected dogs at four times points (0, 12, 25, 26 weeks) and from the five *H. felis*-infected dogs at 25 weeks postinfection. Two microliters from a 1 : 10 dilution of DNA was added to PCR buffer, 400 μM dNTPs, 2 mM $MgCl_2$ (Gibco BRL), 0.5 μM of each primer, 1.5 units of *Taq* DNA polymerase (Gibco BRL), and distilled $H_2O$ in a total volume of 50 μl. The PCR cycle was the same as that for *H. felis* except that primer annealing was performed at 55 C. A band of 1,200 base pairs was apparent with these primers with DNA from *H. pylori, H. felis, H. bizzozeronii, H. heilmannii, H. fenelliae, H. bilis, H. cinaedii, H. hepaticus,* and *H. canis.* This band was absent with DNA from *C. jejuni* and *Proteus mirabilis.*

Southern blot analysis was performed as previously described.[4] The PCR amplification product was run on a 1.5% agarose gel, stained with ethidium bromide, denatured in denaturing buffer (1.5 M NaCl, 0.5 M NaOH) for 1 hour, neutralized, renatured in buffer (1 M Tris-HCl, pH 8.2, 1.5 M NaCl), and transferred to a nitrocellulose membrane. The oligonucleotide (5′-GGA-ATA-AGC-GLA-TCT-3′) was 3′-oligolabeled with a nonradioactive labeling kit (ECL 3′-oligolabeling system, Amersham, Little Chalfont, Buckinghamshire, England). Southern blot hybridization and detection were performed as described by the manufacturer.

### Serology

Serum samples collected at 0, 12, and 24 weeks after infection were evaluated by kinetic enzyme-linked immunosorbent assay (ELISA). High-molecular-weight cell-associated protein, purified from a detergent extraction of *H. felis* ATCC 49179 as previously described,[11] was used to coat ELISA plates at 1 μg/well (Enteric Products, Stony Brook, NY). Serum diluted 1 : 100 in PBS with 0.05% Tween 20 was added to the wells (100 μl/well) and incubated for 1 hour at 37 C. Plates were washed four times in a plate washer (Dynatech, Chantilly, VA) with PBS and 0.05% Tween 20. Bound IgG was detected using horseradish peroxidase goat anti-dog IgG (Cappel/ICN, Costa Mesa, CA) diluted 1 : 2,000 in PBS with 0.05% Tween 20, and 2% dried milk, incubated for 30 minutes followed by washing and incubation with 3,3′,5,5′-teramethylbenzidine Dihydrochloride (TMB). Plates were read immediately after the addition of TMB with a Dynatech MRX plate reader. The plates were read (650 nm) three times at 45-second intervals, with 30 seconds of shaking between readings. The results were expressed as optical density/minute.

### Statistical analysis

Two-way analysis of variance was conducted to determine the effects of group (infected, noninfected) and time on meal-stimulated gastrin release (integrated response determined by calculating the area under the curve) and acid output (basal period pH; pentagastrin-stimulated peak 15-minute period - pH, ml/hour, mmol HCl/ml, mmol HCl/hour, ml/$kg^{0.75}$/hour, mmol HCl/$kg^{0.75}$/hour) before and after administration of *H. felis.* Where significant effects ($P < 0.05$) of time or group were detected, Tukey's test was used to determine which time points were different. Fasting gastrin concentration was evaluated by regression analysis. Difference in the number of immunoreactive somatostatin and gastrin cells in infected and uninfected dogs was evaluated with Student's *t*-test. Correlations for inflammation, lymphoid follicles, and the number of organisms in gastric biopsies were assessed using Spearman's correlation coefficient. All statistical analyses were performed using software (SAS Institute, Cary, NC).

## Results

### Clinical signs

No abnormal clinical signs were evident in the infected and uninfected dogs throughout the study.

### Gross appearance of gastric mucosa

No gross mucosal abnormalities were observed in any dog during upper gastrointestinal endoscopy at 0, 12, or 25 weeks or at necropsy at 26 weeks.

### Infection with *H. felis*

Gastric spiral organisms consistent with *H. felis* were visualized in modified-Steiner-stained sections or impression smears in 4/5 dogs at 12 weeks, 5/5 dogs at 25 weeks, and 4/5 dogs at 26 weeks (Table 1, Fig. 1). Transmission electron microscopy confirmed the presence of spiral bacteria with periplasmic fibrils, consistent with *H. felis* (Fig. 2).[30] A variety of bacteria other than HLO were readily apparent in impression smears. When the density and site of colonization by HLO were assessed, it was apparent that three of the five infected dogs were more heavily colonized than were the other two and that the body, fundus, and cardia were more densely colonized than was the pyloric antrum (Table 2). The degree of colonization visualized at 12 and 25 weeks was similar to that observed at 26 weeks. HLO were most frequently observed in the superficial gastric mucus layer but were also observed within gastric glands and parietal cells (Fig. 1). HLO were not visualized in biopsy samples from the three uninfected dogs at any time.

Biopsy urease tests were positive in 4/5 dogs at 12 and 25 weeks and 3/5 dogs at 26 weeks (Table 1). Urease tests on endoscopic biopsies were more frequently positive in the cardia (8/10 evaluations) and body (7/10 evaluations) than in the pyloric antrum (2/10 evaluations). Urease mapping at 26 weeks confirmed that the cardia, fundus, and body were generally more heavily colonized than was the pyloric antrum (Table 3), though one dog (No. 5) was urease positive at all sites. One biopsy from an uninfected dog was

**Table 1.** *H. felis* colonization in dogs determined by the presence (+) or absence (−) of urease production, gastric spiral organisms (IS = impression smears; MS = modified Steiner stain), and *H. felis* DNA (PCR) in gastric biopsies. The pooled results are from biopsies taken from the pyloric antrum, body, and cardia/fundus. Biopsies at 12 and 25 weeks were obtained by endoscopy. Biopsies at 26 weeks were obtained at necropsy.

| | 12 weeks | | | | 25 weeks | | | | 26 weeks | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Dog No. | IS | MS | Urease | PCR | IS | MS | Urease | PCR | MS | Urease | PCR |
| *H. felis* infected | | | | | | | | | | | |
| 1 | − | − | − | − | + | − | − | + | − | − | + |
| 2 | + | + | + | + | + | + | + | + | + | + | + |
| 3 | + | + | + | + | + | + | + | + | + | + | + |
| 4 | + | + | + | + | − | + | + | − | + | − | + |
| 5 | + | + | + | + | + | + | + | + | + | + | + |
| Control | | | | | | | | | | | |
| 6 | − | − | − | − | − | − | − | − | − | − | − |
| 7 | − | − | − | − | − | − | − | − | − | − | − |
| 8 | − | − | − | − | − | − | − | − | − | + | − |

urease positive at 26 weeks. No other biopsies were positive in this dog at any other time, and spiral organisms were not detected in any biopsies from this dog at any time.

PCR of gastric biopsies using primers for *H. felis* urease was positive in biopsies from 4/5 dogs at 12 and 25 weeks and 5/5 dogs at 26 weeks (Table 1, Fig. 3). In biopsies obtained at 26 weeks, PCR was positive in 5/5 biopsies from the gastric body, 4/5 from the cardia, and 0/5 from the pylorus (Table 2). Southern blot analysis with a labeled oligonucleotide probe for the *H. felis* urease B gene confirmed that PCR products were specific (Fig. 3). PCR of gastric biopsies using *Helicobacter*-specific primers was positive in biopsies from 5/5 *H. felis*-infected dogs at 25 weeks (Fig. 4). No positive PCR results were obtained using either the *H. felis*- or *Helicobacter* genus-specific primers in any of the uninfected dogs at any time. Attempts to culture *H. felis* from gastric biopsies were unsuccessful.

**Gastric histopathology**

No lymphoid follicles and only one area of mild inflammation (grade 1) in one dog were detected in biopsies taken from infected and control dogs before entry to the study. The degree of inflammation was lowest in both groups of dogs at baseline and tended to increase from baseline to 12 weeks and then remained the same at 25 and 26 weeks. Blinded evaluation of biopsy specimens revealed that the degree of inflammation was similar in infected and uninfected dogs (Table 4). In sections examined at 26 weeks, the pyloric antrum was clearly more inflamed than the cardia and fundus (Table 4). Inflammation consisted of plasma cells with rare lymphocytes (Fig. 5). Neutrophils and eosinophils were not involved. There was no relationship between the degree of inflammation and the degree of bacterial colonization. Lymphoid follicles were observed in biopsies from uninfected and

**Fig. 1.** Gastric body; dog No. 3. Note the numerous spiral organisms in the superficial gastric mucus. Modified Steiner stain. Bar = 16 μm.

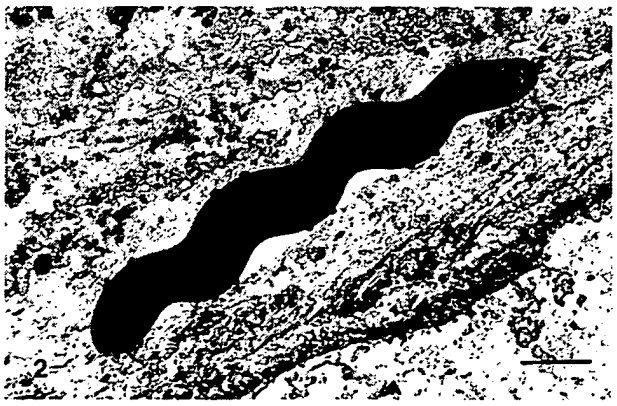

**Fig. 2.** Electron micrograph. Gastric biopsy; *H. felis*-infected dog. Note the spiral shape and distinctive periplasmic fibrils. Bar = 666.7 nm.

**Table 2.** Colonization of different areas of the canine stomach by *H. felis* 26 weeks after infection as determined by modified-Steiner-stained sections (MS), urease mapping, and PCR with *H. felis* primers.

| Dog No. | Pyloric Antrum | | | Body | | | Cardia/Fundus | | |
|---|---|---|---|---|---|---|---|---|---|
| | MS | Urease | PCR | MS | Urease | PCR | MS | Urease | PCR |
| *H. felis* infected | | | | | | | | | |
| 1 | − | − | − | − | − | + | − | − | + |
| 2 | + | − | − | + | + | + | + | + | + |
| 3 | + | − | − | + | + | + | + | + | + |
| 4 | − | − | − | + | − | + | − | − | − |
| 5 | + | + | − | + | + | + | + | + | + |
| Control | | | | | | | | | |
| 6 | − | − | − | − | − | − | − | − | − |
| 7 | − | − | − | − | − | − | − | − | − |
| 8 | − | − | − | − | − | − | − | + | − |

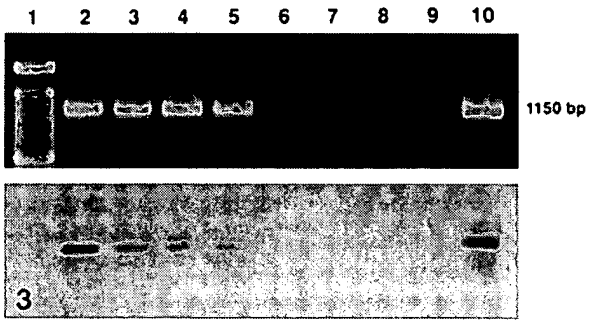


**Fig. 3.** Detection of *H. felis* DNA in endoscopic gastric biopsies by PCR (upper bands) and confirmation with Southern blot (lower bands). Lane 1 = DNA ladder; lane 2 = dog No. 5, 12 weeks; lane 3 = dog No. 4, 12 weeks; lane 4 = dog No. 3, 12 weeks; lane 5 = dog No. 2, 12 weeks; lane 6 = dog No. 1, 12 weeks; lane 7 = dog No. 7, 12 weeks; lane 8 = dog No. 6, 25 weeks; lane 9 = dog No. 8, 25 weeks; lane 10 = DNA from *H. felis* ATCC 49179.

infected dogs and tended to be more numerous in the pyloric antrum than in the body and cardia (Fig. 6, Table 4).

### Acid secretion

Gastric secretion during the basal period was viscous and low in volume, and titratable acidity could not be reliably determined. The pH of basal gastric secretion, measured in the 0–30-minute unstimulated collection period, varied widely among dogs, ranging from pH 2.2 to pH 7.7. There was no significant difference in basal pH ($P > 0.05$) within or between the uninfected and infected dogs at any time (Fig. 7). Pentagastrin-stimulated acid output was maximal during the 60–75-minute period. Secretion volume showed a tendency to decrease from time 0 to weeks 12 and 25, and body-weight-adjusted volume (ml/kg$^{0.75}$/hour) was significantly lower ($P < 0.02$) at 25 weeks than at baseline (Fig. 7). This trend was similar in infected and uninfected dogs. The acidity of gastric secretion during maximal output (pH, mmol HCl/ml) was similar in both groups throughout the study. Total acid output (mmol/kg$^{0.75}$/hour) was similar in infected and uninfected dogs but was significantly higher ($P < 0.01$) at week 0, than at weeks 12 and 25 (Fig. 7).

### Plasma gastrin

There was no significant difference in fasting or meal-stimulated gastrin concentrations within or between groups over the 24-week period (Fig. 8, 9).

### Somatostatin and gastrin immunoreactivity in gastric biopsies

The numbers of immunoreactive somatostatin and gastrin cells and ratio of gastrin : somatostatin cells in

**Table 3.** Urease activity of canine gastric biopsies obtained from 10 standardized sites.[31] The time taken for a color change in the indicator solution from red to pink was recorded.*

| Dog No. | Cardia | Fundus | | Body | | | Pylorus | | | Pyloric Canal |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| *H. felis* infected | | | | | | | | | | |
| 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | ++ | ++ | ++ | ++ | + | ++ | 0 | 0 | 0 | 0 |
| 3 | ++ | +++ | +++ | +++ | +++ | ++ | 0 | 0 | 0 | 0 |
| 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | +++ | +++ | +++ | +++ | +++ | +++ | ++ | + | + | ++ |
| Control | | | | | | | | | | |
| 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 8 | 0 | ++ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

* +++ = <4 hours; ++= <24 hours; + = <48 hours; 0 = negative after 48 hours.

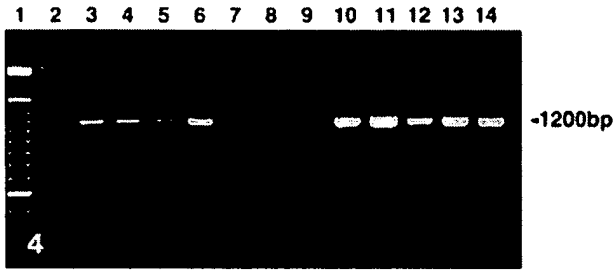


**Fig. 4.** Detection of *Helicobacter* spp. DNA in endoscopic gastric biopsies by PCR. Lane 1 = DNA ladder; lane 2 = dog No. 1; lane 3 = dog No. 2; lane 4 = dog No. 3; lane 5 = dog No. 4; lane 6 = dog No. 5; lane 7 = dog No. 6; lane 8 = dog No. 7; lane 9 = dog No. 8; lane 10 = DNA from *H. felis*; lane 11 = DNA from *H. bizzozeronii*; lane 12 = DNA from *H. heilmannii;* lane 13 = DNA from *H. pylori;* lane 14 = DNA from *H. bilis.*

the antrum was similar in infected and uninfected dogs, using either of the cell counting methods (Table 5).

### Serology

ELISA results are shown in Fig. 10. Three of the infected dogs (Nos. 2, 4, 5) showed evidence of seroconversion, with progressive increases in optical density/minute to 2.5–5 times baseline values at 25 weeks. Two of the uninfected dogs (Nos. 6, 7) showed little change in optical density/minute throughout the 25-week period; however, one uninfected dog (No. 8) showed a gradual increase to 2.5 times baseline at 25 weeks.

## Discussion

The oral administration of *H. felis* resulted in the colonization of 5/5 dogs. Infection was achieved solely by administering a suspension of *H. felis.* It was not necessary to suppress gastric acid secretion or use gnotobiotic dogs.[13,31] Although *H. felis* was not cultured from gastric biopsies of infected dogs, the presence of HLO with periplasmic fibrils revealed by electron microscopy and positive PCR results using *H. felis*-specific primers indicates that the gastric HLO observed in biopsies were *H. felis.*[31,39] Uninfected dogs were negative for *Helicobacter* spp. throughout the study.

The combination of urease mapping, histopathology, and PCR results indicated that the cardia, fundus, and body were more heavily colonized than the pyloric antrum. This pattern of colonization was similar to that observed in gnotobiotic dogs infected with *H. felis* and dogs with naturally acquired gastric helicobacteriosis.[24,31] The density of colonization determined by urease mapping in SPF dogs appeared lower than that in gnotobiotic dogs infected with *H. felis,* where biopsies from all sites were urease positive at 24 hours, and in dogs with naturally acquired gastric helicobacteriosis, where biopsies are usually urease positive within 2–4 hours.[23,31] The biopsy urease test was positive in one

**Table 4.** Histopathologic findings from gastric biopsies* 26 weeks after dogs were infected with *H. felis.*

| Dog No. | Colonization Density† | | | Inflammation‡ | | | No. Lymphoid Follicles | | |
|---|---|---|---|---|---|---|---|---|---|
| | P | B | C | P | B | C | P | B | C |
| *H. felis* infected | | | | | | | | | |
| 1 | 0 | 0 | 0 | 2 | 0 | 0 | 3 | 1 | 0 |
| 2 | 2 | 1 | 2 | 1 | 0 | 1 | 1 | 2 | 0 |
| 3 | 1 | 3 | 2 | 1 | 0 | 0 | 0 | 0 | 0 |
| 4 | 0 | 1 | 0 | 2 | 0 | 0 | 1 | 1 | 1 |
| 5 | 1 | 3 | 3 | 2 | 1 | 0 | 0 | 1 | 0 |
| Control | | | | | | | | | |
| 6 | 0 | 0 | 0 | 2 | 0 | 0 | 1 | 0 | 0 |
| 7 | 0 | 0 | 0 | 2 | 1 | 0 | 3 | 0 | 0 |
| 8 | 0 | 0 | 0 | 2 | 0 | 0 | 4 | 2 | 0 |

* P = pyloric antrum; B = body; C = cardia/fundus.
† Scale of 0–3.
‡ Scale of 0–2.

**Fig. 5.** Pyloric antrum; dog No. 6. Uninfected dog with moderate gastritis (grade 2), 26 weeks. HE. Bar = 80 μm.

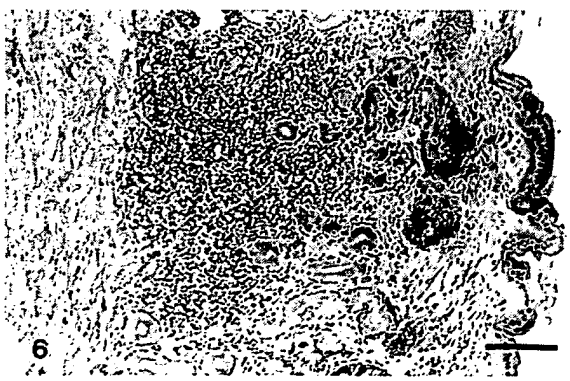


**Fig. 6.** Pyloric antrum; dog No. 6. Prominent lymphoid follicle, 26 weeks. HE. Bar = 120 μm.

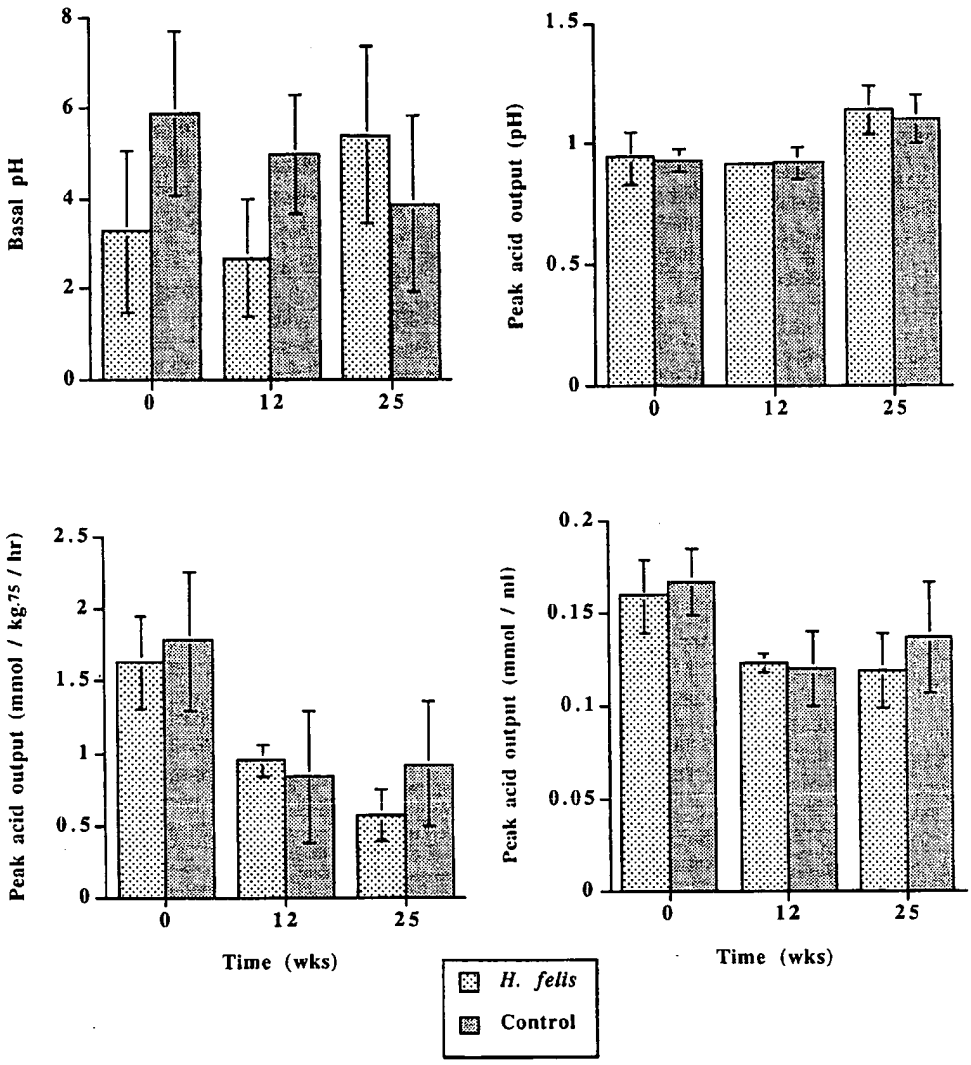


**Fig. 7.** Gastric acid secretion in dogs (mean ± SE) before and after infection with *H. felis*.

biopsy from an uninfected dog (No. 8), which could be related to urease production by bacteria other than *Helicobacter* spp. *Proteus mirabilis,* a strong urease producer, also has been cultured from the gastric mucosa of coprophagic SPF Beagles in our colony (unpublished observation).

A number of methods were used to detect *H. felis* in gastric biopsies. When the results of biopsies taken from infected dogs at weeks 12, 25, and 26 are combined, it is apparent that PCR was positive for *H. felis* at 13 of 15 sampling points, impression smears and modified-Steiner-stained smears were positive for gastric HLO at 8 of 10 and 12 of 15 sampling points, respectively, and urease tests was positive at 11 of 15 sampling points. In the less heavily colonized dogs, impression smears, Steiner-stained sections, and PCR results appeared more sensitive than biopsy urease tests. These observations concur with those of *H. felis*-infected mice and of dogs with naturally acquired helicobacteriosis, where microscopy was more sensitive than culture and urease mapping.[23,35] The identification of *Helicobacter* spp. in biopsies from dogs using PCR has not been reported previously and has implications for the detection of an identification to species of *Helicobacter* spp. in dogs with naturally occurring gastric helicobacteriosis. Our observation that PCR was sensitive and specific is in agreement with studies in mice infected with *H. felis* and in humans and cats infected *H. pylori,* which showed that PCR was more sensitive than histology, bacterial culture, and urease mapping.[12,28,40] Although the present study suggests that PCR is the most sensitive test for the detection of *H. felis* in dogs, PCR on pyloric biopsies taken from dogs at 26 weeks was negative, despite evidence of spiral

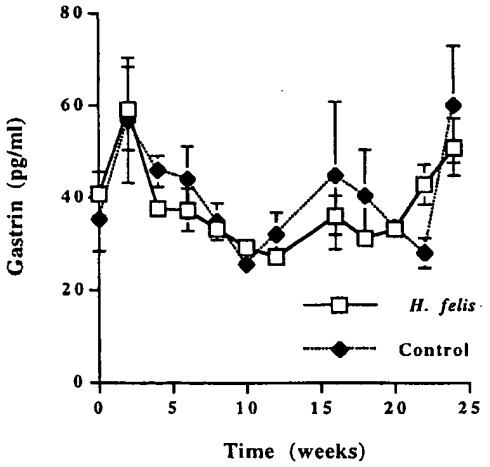


**Fig. 8.** Fasting plasma gastrin in dogs (pg/ml; mean ± SE) before and after infection with *H. felis*.

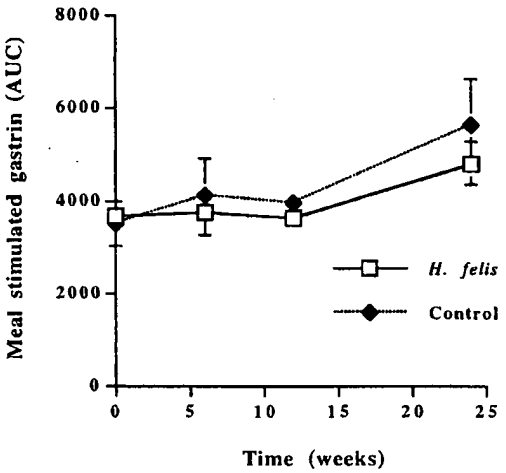


**Fig. 9.** Meal-stimulated plasma gastrin in dogs (mean ± SE; integrated response, calculated by measuring the area under the curve [AUC]) before and after infection with *H. felis*.

organisms on histopathology. The patchy distribution of *Helicobacter* within the stomach, particularly in dogs with low colonization density, may account for this finding and indicates that the confirmation of *Helicobacter* infection is probably best undertaken by evaluating multiple biopsies from different sites by a combination of methods.

There was no relation between *H. felis* colonization or colonization density and the severity of mucosal inflammation or the number of lymphoid follicles observed. The degree of inflammation and number of lymphoid follicles increased in both groups from baseline to 12 weeks and then stabilized. Because the degree of inflammation was similar in infected and uninfected dogs, it may be related to a natural age-related change or perhaps repeated endoscopic biopsy. The pyloric antrum was the area with most inflammation and lymphoid follicles, despite being least densely colonized with HLO. This finding is similar to observations in dogs with naturally acquired gastric helicobacteriosis, where plasma cell inflammation was most marked in the antrum, despite lower colonization at this site.[24] The present findings suggest that the more marked inflammation detected in the pyloric antrum of those dogs with naturally occurring helicobacteriosis may not be *Helicobacter* related. These findings contrast with those in gnotobiotic dogs infected with *H. felis,* where lymphoid follicle hyperplasia and bacterial colonization were most evident in the fundus and body, and subglandular infiltrates of lymphocytes, plasma cells, and eosinophils were widespread.[31] Conversely, *H. felis*-infected mice have more inflammation in the fundus than in the pyloric antrum, although the pyloric antrum is generally more densely colonized.[35] The species and strain of the host determines the density of bacterial colonization and the degree and type

**Table 5.** Number of immunoreactive somatostatin (SMS) and gastrin (G) cells in biopsies from the pyloric antrum in dogs. Cells were counted by two different methods (1 and 2).

| | Method 1 | | | Method 2 | | |
|---|---|---|---|---|---|---|
| Dog No. | SMS | G | G:SMS | SMS | G | G:SMS |
| *H. felis* infected | | | | | | |
| 2 | 81.8 | 500 | 6.1 | 4.8 | 24.8 | 5.2 |
| 3 | 141.8 | 456 | 3.2 | 8.2 | 21 | 2.6 |
| 4 | 83 | 247 | 3 | 3.5 | 15.4 | 4.4 |
| 5 | 121.2 | 291 | 2.4 | 6.3 | 16.5 | 2.6 |
| Mean ± SD | 106.7 ± 29.2 | 373 ± 123 | 3.7 ± 1.7 | 5.7 ± 2 | 19.4 ± 4.3 | 3.7 ± 1.3 |
| Control | | | | | | |
| 6 | 96.6 | 439 | 4.5 | 4.9 | 21.8 | 4.4 |
| 7 | 84.7 | 309 | 3.7 | 5.2 | 14.4 | 2.8 |
| Mean ± SD | 90.7 ± 8.4 | 374 ± 91 | 4.1 ± 0.6 | 4.8 ± 5 | 21.8 ± 14.4 | 3.6 ± 1.1 |

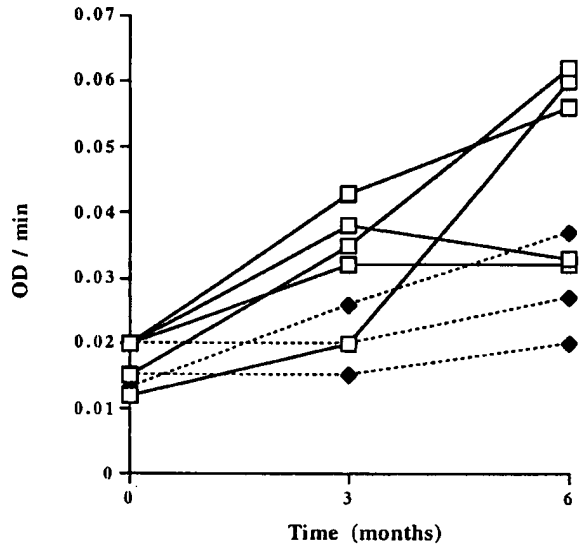


**Fig. 10.** Serologic response (IgG) of SPF dogs to infection with *H. felis* (□) and uninfected dogs (♦). ELISA results are expressed as optical density (OD)/minute.

of inflammation observed in response to infection with *H. felis*.[17,35] Differences in the density of bacterial colonization and mucosal inflammation in *H. felis*-infected SPF and gnotobiotic dogs may therefore reflect the age and origins of the dogs. The apparently higher degree of *H. felis* colonization in gnotobiotic dogs may be related to lack of competition from other gastric bacteria, a situation that was clearly evident in SPF dogs. Additionally, basal and pentagastrin-stimulated acid secretion may not be fully developed in Beagles until 5 weeks of age.[33] Thus, lower acidity in gnotobiotic than in SPF dogs may have enabled bacterial colonization because inhibiting acid secretion is thought to facilitate colonization of the gastric mucosa.[13] Differences in the immunologic response of 7-day-old gnotobiotic bacteria-free dogs versus 4-month-old SPF dogs when exposed to a variety of bacterial antigens may also have had an effect on the degree of colonization and inflammation. Although the strain of *H. felis* used was the same in both SPF and gnotobiotic dogs, an effect of passage or loss of virulence cannot be discounted.

Seroconversion was observed in 3/5 infected dogs, and optical density/minute gradually increased to values 2.5–5 times higher than baseline after 6 months of infection. Previous studies of *H. felis* infection in gnotobiotic dogs and mice have demonstrated fairly uniform seroconversion 2 weeks after infection.[14,31] The variable serologic responses observed here are more similar to those in cats infected with *H. pylori*, where some cats seroconverted within 2 weeks and peaked at 8 times baseline, whereas others did not seroconvert until 6 months after infection and had titers only two-fold greater than baseline.[13] In the present study, seroconversion did not appear related to colonization density or inflammation because the second most heavily colonized dog (No. 3) did not seroconvert, whereas one of the least heavily colonized dogs did (dog No. 4). The reason for the differences in the time and degree of seroconversion in *H. pylori*-infected cats was similarly unclear, and the degree of bacterial colonization and number of lymphoid follicles were comparable in all of the cats.[13] One uninfected dog showed evidence of seroconversion, with a gradual increase in optical density/minute to 2.5 times baseline at 25 weeks. This dog (No. 8) had one urease-positive biopsy at week 26. Although no HLO were detected in this dog at any time, seroconversion may have been related to an immune response against another urease producing bacterium, such as *Proteus mirabilis*. The similarity in fasting and meal-stimulated plasma gastrin, basal and pentagastrin-stimulated gastric acid secretion, and immunoreactive somatostatin : gastrin cells in infected and uninfected dogs suggests that colonization with *H. felis* for 6 months does not perturb the gastric secretory axis. The *H. felis*-infected dogs in the present study were asymptomatic, with normal stimulated acid output, similar to asymptomatic people infected with *H. pylori*, who have normal gastrin-stimulated acid output.[8,20] However, basal and meal-stimulated hypergastrinemia and decreased immunoreactive somatostatin cells, which are encountered in people infected with *H. pylori*, were absent in *H. felis*-infected dogs.[8,20,38,41] The initial phase of *H. pylori* infection in people has been associated with achlorhydria, which is often transient.[8,34,36,45] Achlorhydria associated with *H. pylori* infection is attributed to inhibition of gastric secretion by the organism, the inflammatory response induced in the mucosa, and gastric atrophy.[3,8,47] It has been suggested, on the basis of high unstimulated gastric pH, that *H. felis* and *H. pylori* induce achlorhydria in gnotobiotic dogs.[31,43] The marked variability of unstimulated gastric pH observed in the present study is similar to that seen in gnotobiotic dogs infected with *H. felis* and *H. pylori* and in dogs of unknown *Helicobacter* infection status.[22,31,43] The present study and a previous one of dogs of unknown *Helicobacter* infection status demonstrate that dogs with high unstimulated gastric pH are able to produce similar amounts of acid in response to pentagastrin stimulation.[22] Thus, inferences about the ability of *H. pylori* and *H. felis* to induce achlorhydria in 4/10 gnotobiotic dogs, based on the measurement of unstimulated gastric pH, are not justified.[31,43] *H. felis* may have induced transient achlorhydria in SPF dogs that was missed at gastric acid evaluation at 3 months; however, this explanation seems unlikely because fast-

ing and meal-stimulated gastrin were similar throughout.

The reason for the drop in stimulated acid output in uninfected and infected dogs at 12 weeks is unclear, but it may reflect maturation of gastrin release and acid secretion, although this is reported to be fully mature in Beagles by 5 weeks.[33] The stimulated acid output reported here is lower than that observed in a previous study of dogs of unknown *Helicobacter* infection status, but the acidity of the gastric secretion (mmol HCl/ml) is similar in both studies, indicating that decreased volume was the reason for the lower acid output in the present study.[22] This lower acid output may reflect differences in anesthetic technique, the route and dose of pentagastrin stimulation, of the *Helicobacter* status of the dogs.

Although we failed to detect a significant pathologic change in our experimental dogs, the time scale of the experiment, 6 months, was relatively short when compared with the length of time pet dogs are colonized by gastric *Helicobacter* spp. Future studies with a longer infection time, to mimic the natural course of infection and parallel infection of people with *H. pylori*, coupled with a more robust *H. felis* infection may be necessary to determine the relationship of *H. felis* infection to gastritis in dogs.

## Acknowledgements

We thank Enteric Products Inc., Stony Brook, NY, for supplying *H. felis* and antigen-coated ELISA plates, Brent Howe, Ohio State University, for gastrin analysis, Tina Smith for immunostaining, Anita Alisio and Shannon Caldwell for electron microscopy, Hussni Mohammed for statistical advice, Reto Neiger for PCR primer sequences, and Leland Carmichael for his help and advice throughout the study.

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Request reprints from Dr. K. W. Simpson, Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853 (USA). E-mail: KWS5@cornell.edu.

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# Role of Helicobacter felis in chronic canine gastritis.

**Lee A, Krakowka S, Fox JG, Otto G, Eaton KA, Murphy JC.**

School of Microbiology, University of New South Wales, Kensington, Australia.

Five gnotobiotic Beagle dogs were orally inoculated with a pure culture of Helicobacter felis. The remaining two littermates served as contact controls. Thirty days after infection, all animals were euthanatized and specimens were collected for evaluation. In infected dogs, H. felis was recovered from all areas of the stomach. Colonization was heaviest in the fundus and antrum. H. felis was not cultured from any segment of the gastrointestinal tract distal to the duodenum. Two weeks after infection, all five infected dogs had detectable IgM and IgG serum antibody to H. felis, whereas control dogs had no measurable H. felis serum antibody throughout the study. Histopathologic changes in the stomachs of infected dogs included large numbers of lymphoid nodules throughout all regions of the gastric mucosa and were most numerous in the fundus and body. A mild, diffuse lymphocytic infiltrate with small numbers of plasma cells and eosinophils was also present in the subglandular region of all portions of the gastric mucosa. Electron microscopic examination revealed large numbers of spiral-shaped H. felis in gastric mucus adjacent to or superimposed over the areas of inflammation. Occasionally, however, H. felis was observed within the canaliculi of gastric parietal cells. Histopathologic changes in the stomachs of the contact control dogs were limited to focal infiltrates of eosinophils and small aggregates of lymphocytes in the subglandular portions of the gastric mucosa in one animal. Infection with H. felis is a likely cause of naturally occurring lymphofollicular gastritis.

PMID: 1448894 [PubMed - indexed for MEDLINE]

# WEST Search History

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DATE: Monday, November 26, 2007

| Hide? | Set Name | Query | Hit Count |
|---|---|---|---|
| | | *DB=PGPB,USPT,USOC,EPAB,JPAB,DWPI; PLUR=YES; OP=OR* | |
| ☐ | L12 | L11 and L1 | 4 |
| ☐ | L11 | (cattoli or kusters!).in. | 477 |
| ☐ | L10 | L9 and (cs-1 or cs1) | 4 |
| ☐ | L9 | L7 same (helico or helicobacter or helico-bacter or hfelis or h-felis) | 81 |
| ☐ | L8 | L7 and (helico or helicobacter or helico-bacter or hfelis or h-felis) | 102 |
| ☐ | L7 | ($felis! same $urease) | 102 |
| ☐ | L6 | L5 and ($felis! same $urease) | 29 |
| ☐ | L5 | L4 and l1.ti,ab,clm. | 31 |
| ☐ | L4 | L3 and urease.ti,ab,clm. | 55 |
| ☐ | L3 | L2 and (helico or helicobacter or helico-bacter or hfelis or h-felis) | 153 |
| ☐ | L2 | L1 and $urease | 185 |
| ☐ | L1 | $felis! | 2073 |

END OF SEARCH HISTORY

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DOCUMENT-IDENTIFIER: US 20030180330 A1
TITLE: Method for identifying helicobacter antigens

search note

Detail Description Paragraph:
[0242] 60. Michetti, P., Corthesy-Theulaz, I., Davin, C., Haas, R., Vaney, A. C., Heitz, M., Bille, J., Kraehenbuhl, J. P., Saraga, E., and Blum, A. L. (1994) Immunization of BALB/c mice against Helicobacter felis infection with Helicobacter pylori urease. Gastroenterology 107: 1002-1011.

Detail Description Paragraph:
[0279] 97. Lee, C. K., Weltzin, R., Thomas, W. D. J., Kleanthous, H., Ermak, T. H., Soman, G., Hill, J. E., Ackerman, S. K., and Monath, T. P. 1995. Oral immunization with recombinant Helicobacter pylori urease induces secretory IgA antibodies and protects mice from challenge with Helicobacter felis. J Infect Dis 172: 161-172.

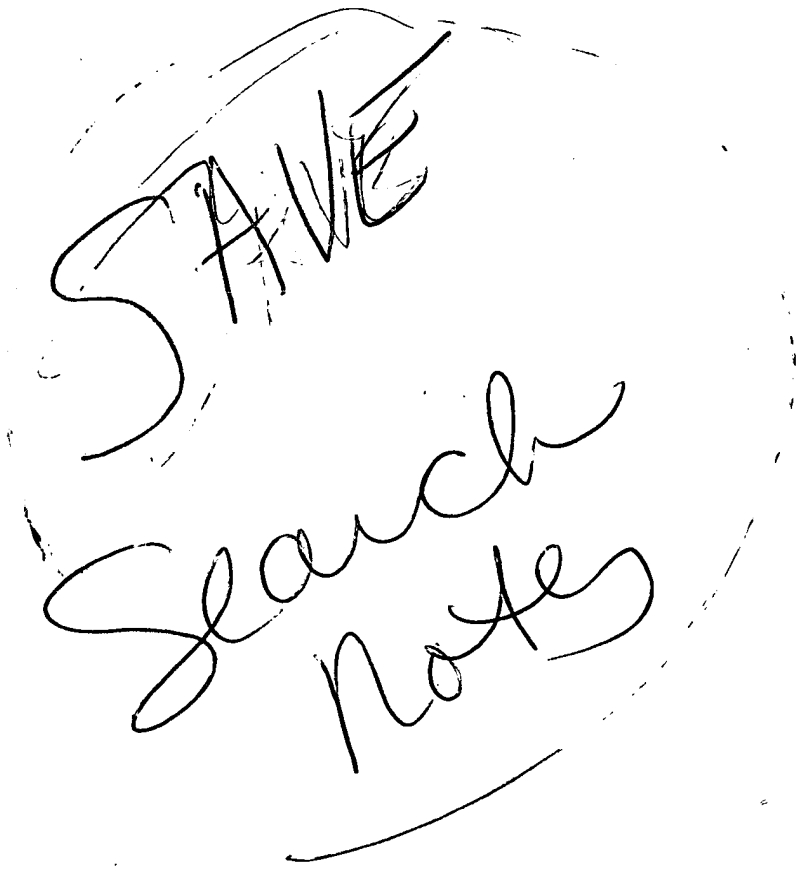

TABLE 2-continued

Sources and accession numbers of strains studied

| Organism | Strain examined[a] | Culture Collections[b] | GenBank[c] accession no. |
|---|---|---|---|
| *H. hepaticus* | Fox Hh-2 ™ | ATCC 51448 ™ | U07574 |
| *H. hepaticus* | Fox Hh-3 | ATCC 51450 | U07575 |
| Reference species | | | |
| *A. cryophilus* | CCUG 17801 ™ | ATCC 43158 ™ | L14624 |
| *A. butzleri* | CCUG 10373 | | L14626 |
| *A. skirrowii* | CCUG 10374 ™ | | L16625 |
| *C. coli* | CCUG 11238 ™ | ATCC 33559 ™ | L04312 |
| *C. concisus* | Tanner 484 ™ | ATCC 33237 ™ | L04322 |
| *C. fetus ss fetus* | ATCC 27374 ™ | | M65012 |
| *C. lari* | CCUG 23947 ™ | ATCC 35221 ™ | L04316 |
| *C. rectus* | Tanner 371 ™ | ATCC 33238 ™ | L04317 |
| "*F. rappini*" | NADC 1893 ™ | ATCC 43966 ™ | M88137 |
| "*Gastrospirillum hominis* 1" | | | L10079 |
| "*Gastrospirillum hominis* 2" | | | L10080 |
| *H. acinonyx* | Eaton 90-119-3 ™ | ATCC 51101 ™ CCUG 29263T ™ | M88148 |
| *H. canis* | NCTC 12739 ™ | | L13464 |
| *H. cinaedi* | CCUG 18818 ™ | ATCC 35683 ™ | M88150 |
| *H. felis* | Lee CS1 ™ | ATCC 49179 ™ | M37642 |
| *H. fennelliae* | CCUG 18820 ™ | ATCC 35684 ™ | M88154 |
| *H. mustelae* | Fox R85-13-6 ™ | ATCC 43772 ™ | M35048 |
| *H. muridarum* | Lee ST1 ™ | CCUG 29262 ™ ATCC 49282 ™ | M80205 |
| *H. nemestrinae* | — | ATCC 49396 ™ | X67854 |
| *H. pametensis* | Seymour B9 ™ | CCUG 29255 ™ | M88147 |
| *H. pylori* | ATCC 43504 ™ | | M88157 |
| Helicobacter sp. "CLO-3" (human) | CCUG 14564 | LMG 7792 | M88151 |
| Helicobacter sp. "B" (bird) | Seymour B10 ™ | CCUG 29256 ™ | M88139 |
| Helicobacter sp. "C" (bird) | Seymour B52 ™ | CCUG 29561 ™ | M88144 |
| *W. succinogenes* | Tanner 602W ™ | ATCC 29543 ™ | M88159 |

[a]Stains from which sequences were determined were obtained from the following individuals or culture collections: K. A. Eaton, Department of Veterinary Pathobiology, Ohio State University, Columbus, Ohio; J. G. Fox, Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, Mass.; A. Lee, Department of Microbiology and Immunology, University of New South Wales, Sydney; C. Seymour, Department of Microbiology, Boston University School of Medicine, Boston, Mass.; A. Tanner, Department of Microbiology, Forsyth Dental Center, Boston, Mass; ATCC, American Type Culture Collection, Rockville, Maryland; CCUG, Culture Collection, University of Göeteborg, Göeteborg, Sewden; LMG, Laboratorium voor Microbiologie en microbielle Genetica, Ghent, Belgium; NADC, National Animal Disease Center, Ames, Iowa; or NCTC, National Collections of Type Cultures, London, United Kingdom.

[b]Alternate culture collection sources for sequenced strains. Abrevations as above.

[c]16S rRNA sequences for these strains are available for electronic retrieval from GenBank under the following accession numbers. Through cross distribution of data bases, these sequences should also be available from EMBL and DDBJ.

TABLE 3

Similarity matrix based upon 16S rRNA sequence comparisons[a]

| | *H.he* | *H.mr* | *H.ca* | *F.ra* | *H.ci* | *H.fe* | H.s3 | *H.py* | *H.ne* | *H.ac* | *G.h1* | *G.h2* | *H.fe* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Helicobacter hepaticus* | . | 97.8 | 97.3 | 97.4 | 97.0 | 95.5 | 95.2 | 93.5 | 93.6 | 93.2 | 92.3 | 92.8 | 93.3 |
| *Helicobacter muridarum* | 2.3 | . | 96.5 | 96.1 | 95.9 | 95.1 | 94.3 | 93.1 | 93.1 | 92.5 | 91.9 | 92.4 | 92.6 |
| *Helicobacter canis* | 2.8 | 3.6 | . | 98.0 | 97.8 | 95.4 | 96.0 | 93.9 | 93.8 | 93.2 | 92.3 | 92.7 | 93.2 |
| "*Flexispira rappini*" | 2.6 | 4.0 | 2.0 | . | 98.8 | 95.4 | 95.5 | 93.2 | 93.4 | 92.5 | 92.2 | 92.3 | 92.6 |
| *Helicobacter cinaedi* | 3.1 | 4.2 | 2.3 | 1.2 | . | 95.9 | 95.3 | 92.8 | 93.3 | 92.4 | 92.0 | 92.4 | 92.6 |
| *Helicobacter fennelliae* | 4.6 | 5.1 | 4.7 | 4.8 | 4.3 | . | 94.8 | 93.1 | 92.8 | 92.3 | 92.6 | 92.8 | 93.0 |
| Helicobacter sp. "CLO-3" | 4.9 | 5.9 | 4.1 | 4.6 | 4.9 | 5.4 | . | 93.9 | 93.4 | 93.0 | 92.7 | 92.8 | 93.2 |
| *Helicobacter pylori* | 6.8 | 7.2 | 6.4 | 7.1 | 7.5 | 7.3 | 6.4 | . | 98.2 | 97.4 | 94.9 | 95.1 | 95.4 |
| *Helicobacter nemestrinae* | 6.7 | 7.3 | 6.4 | 6.9 | 7.0 | 7.6 | 6.9 | 1.8 | . | 96.7 | 94.7 | 94.9 | 95.5 |
| *Helicobacter acinonyx* | 7.1 | 8.0 | 7.1 | 7.9 | 8.0 | 8.1 | 7.4 | 2.6 | 3.3 | . | 94.7 | 96.0 | 96.4 |
| "*Gastrospirillum hominis*" 1 | 8.1 | 8.6 | 8.1 | 8.3 | 8.4 | 7.8 | 7.6 | 5.3 | 5.5 | 5.5 | . | 96.5 | 96.6 |
| "*Gastrospirillum hominis*" 2 | 7.6 | 8.0 | 7.6 | 8.1 | 8.0 | 7.6 | 7.5 | 5.1 | 5.3 | 4.1 | 3.6 | . | 98.8 |
| *Helicobacter felis* | 7.0 | 7.8 | 7.1 | 7.7 | 7.8 | 7.3 | 7.1 | 4.7 | 4.7 | 3.7 | 3.4 | 1.3 | . |
| *Helicobacter pametensis* | 3.8 | 4.5 | 3.6 | 3.6 | 4.6 | 4.6 | 5.0 | 5.7 | 5.6 | 6.3 | 7.8 | 6.6 | 6.3 |
| Helicobacter sp. "C" (bird) | 3.6 | 3.8 | 3.2 | 3.7 | 4.5 | 5.6 | 4.7 | 6.1 | 5.7 | 6.8 | 8.2 | 7.5 | 7.1 |
| Helicobacter sp. "B" (bird) | 3.6 | 4.1 | 3.2 | 4.3 | 4.8 | 5.6 | 5.0 | 6.5 | 6.2 | 6.8 | 8.2 | 7.3 | 6.9 |
| *Helicobacter mustelae* | 3.7 | 4.4 | 3.7 | 4.3 | 5.0 | 5.8 | 4.9 | 6.4 | 6.2 | 6.8 | 8.4 | 7.1 | 6.9 |
| *Wolinella succinogenes* | 7.6 | 6.9 | 6.9 | 7.3 | 7.4 | 7.9 | 8.0 | 9.8 | 9.5 | 10.1 | 11.5 | 10.7 | 10.5 |
| *Arcobacter cryaerophilus* | 16.8 | 16.8 | 16.2 | 16.7 | 16.7 | 17.4 | 16.1 | 17.4 | 16.8 | 17.7 | 18.7 | 18.4 | 18.7 |
| *Arcobacter skirrowii* | 17.3 | 17.2 | 16.7 | 17.2 | 17.3 | 17.6 | 16.6 | 18.0 | 17.5 | 18.1 | 19.1 | 19.0 | 18.9 |
| *Arcobacter butzleri* | 15.8 | 15.7 | 15.6 | 16.1 | 16.2 | 16.0 | 15.1 | 16.3 | 16.0 | 16.6 | 18.3 | 17.9 | 17.7 |
| *Campylobacter rectus* | 16.2 | 17.0 | 16.0 | 15.6 | 15.8 | 16.2 | 16.5 | 17.8 | 17.0 | 18.5 | 19.1 | 19.4 | 19.3 |

US005843460A

# United States Patent [19]

Labigne et al.

[11] Patent Number: 5,843,460

[45] Date of Patent: Dec. 1, 1998

[54] **IMMUNOGENIC COMPOSITIONS AGAINST HELICOBACTER INFECTION, POLYPEPTIDES FOR USE IN THE COMPOSITIONS, AND NUCLEIC ACID SEQUENCES ENCODING SAID POLYPEPTIDES**

[75] Inventors: **Agnes Labigne**, Bures S/Yvette, France; **Sebastin Suerbaum**, Bochum, Germany; **Richard L. Ferrero**, Paris; **Jean-Michel Thiberge**, Plaisir, both of France

[73] Assignees: **Institut Pasteur**; **Institut National de la Sante et de la Recherche Medicale**, both of Paris, France

[21] Appl. No.: **467,822**

[22] Filed: **Jun. 6, 1995**

**Related U.S. Application Data**

[63] Continuation of Ser. No. 447,177, May 19, 1995, which is a continuation-in-part of Ser. No. 432,697, May 2, 1995.

[30] **Foreign Application Priority Data**

May 19, 1993 [EP] European Pat. Off. ..... 93 401 309
Nov. 19, 1993 [WO] WIPO ..... PCT/EP93/03259

[51] **Int. Cl.**[6] ..... **A61K 39/02**

[52] **U.S. Cl.** ..... **424/234.1**; 435/7.32; 435/6; 435/7.9; 514/234.5; 514/41

[58] **Field of Search** ..... 435/7.32, 4, 6, 435/7.9; 514/234.5, 41; 424/234.1

[56] **References Cited**

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| | | |
|---|---|---|
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| WOA9109049 | 6/1991 | WIPO . |
| WOA9307273 | 4/1993 | WIPO . |
| WOA9316723 | 9/1993 | WIPO . |
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| WOA9320843 | 10/1993 | WIPO . |
| WOA9406474 | 3/1994 | WIPO . |
| WOA9409823 | 5/1994 | WIPO . |
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| WO 9640893 | 12/1996 | WIPO . |

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R. L. Ferrero et al., "Molecular Evidence Demonstrating Significant Homology Between the Urease Polypeptides of Helicobacter Felis and and Helicobacter Pylori," *Gastroenterology*, vol. 104, No. 4, Apr. 1993, Elsevier, New York, U.S.; p. A699.

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(List continued on next page.)

*Primary Examiner*—James C. Housel
*Assistant Examiner*—Ginny Allen Portner
*Attorney, Agent, or Firm*—Finnegan, Henderson, Farabow, Garrett & Dunner, L.L.P.

[57] **ABSTRACT**

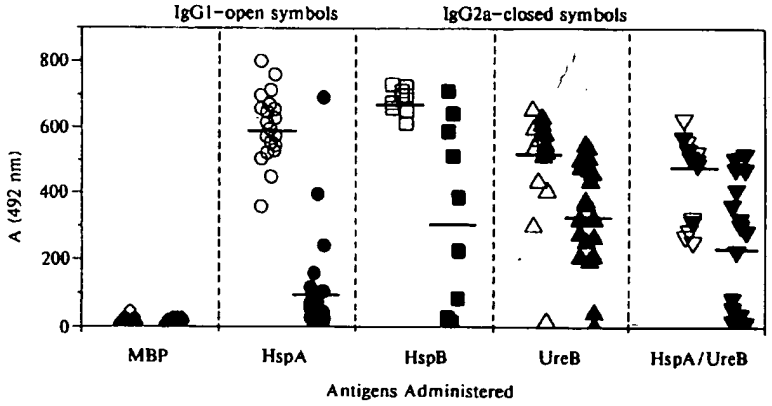
There is provided an immunogenic composition capable of inducing protective antibodies against Helicobacter infection characterized in that it comprises:

i) at least one sub-unit of a urease structural polypeptide from *Helicobacter pylori* (SEQ ID NOS:22,26), or a fragment thereof, said fragment being recognized by antibodies reacting with *Helicobacter felis* urease (SEQ ID NOS:20–21), and/or at least one sub-unit of a urease structural polypeptide from *Helicobacter felis* (SEQ ID NOS:20–21), or a fragment thereof, said fragment being recognized by antibodies reacting with *Helicobacter pylori* urease (SEQ ID NOS:22–26);

ii) and/or, a heat shock protein (Hsp), or chaperonin, from Helicobacter, or a fragment of said protein.

The preparation, by recombinant means, of such immunogenic compositions is also provided.

**10 Claims, 36 Drawing Sheets**



DOCUMENT-IDENTIFIER: US 5837240 A
TITLE: Multimeric, recombinant urease vaccine

Detailed Description Text (46):
Since H. pylori does not readily infect laboratory animals, the H. felis model in rodents was used to test the efficacy of recombinant H. pylori urease in prophylaxis and antibacterial therapy. In this model, colonization of the stomach is readily established and is accompanied by gastric inflammation. This animal model is a well established system for the study of Helicobacter, and has been used extensively in laboratory investigations of the pathogenesis and treatment of Helicobacter-induced disease (Fox et al., Infect. Immun. 61:2309-2315, 1993; Goodwin & Worsley, Helicobacter pylori, Biology and Clinical Practice, CRC Press, Boca Raton, Fla., 465 pp, 1993). Antigenic cross-reactivity between H. pylori and H. felis ureases allows use of the human vaccine candidate of the invention, recombinant H. pylori urease (rUre), to immunize animals infected, or subsequently challenged, with H. felis.

Detailed Description Text (49):
Several methods were used to detect Helicobacter in gastric tissue, including measurement of gastric urease activity, histologic examination, and culture of gastric tissue. Gastric urease activity was measured both qualitatively (presence or absence) and quantitatively. In the qualitative assay, stomachs were divided longitudinally into two halves, from the gastroesophageal sphincter to the pylorus. One longitudinal piece, representing approximately 1/4 of the stomach, was placed in 1 mL of urea broth (0.1 g yeast extract, 0.091 g monopotassium phosphate, 0.095 g disodium phosphate, 20 g urea, and 0.1 g/L phenol red, pH 6.9). A distinctive color change (due to hydrolysis of urea by the enzyme, production of ammonia, and increased pH) after four hours incubation at room temperature indicated a positive result. For quantitative determinations, urease activity was determined by measuring absorbance at 550 nm of clarified urea broth incubated with whole stomach sections for 4 hours. This assay can detect as few as 1-2.times.10.sup.4 H. felis/0.1 g stomach tissue. This assay provides the same sensitivity as commercially available urease test kits used for human samples. Commercial kits have proven to be 100% specific and 90-92% sensitive compared to biopsy/histology (Szeto et al., Postgrad. Med. J. 64:935-936, 1988: Borromeo et al. J. Clin. Pathol. 40:462-468, 1987).

Detailed Description Text (93):
At four weeks, twelve animals receiving LT and 40 animals receiving urease+LT were reinfected with H. felis. Ten weeks after the challenge, the twelve animals receiving LT and 40 animals receiving Urease+LT were reinfected with H. felis. Ten weeks after this challenge, animals were sacrificed to determine the extent of infection by quantitative urease assay. Of the nine animals which were given urease+LT, but not subjected to reinfection, 5 were protected (57%), as determined by gastric urease activity. All twelve LT treated animals which were rechallenged were infected. Thirty seven of the 40 mice (93%) which were given urease+LT, and then re-challenged with H. felis, were protected as determined by reduced gastric urease activity. This experiment shows that urease vaccination not only eradicates an existing Helicobacter infection, but also protects the host against reinfection.

Detailed Description Text (97):
The role of anti-urease antibodies in Helicobacter therapy, i.e., the clearance of H. felis from infected mice, was examined by first infecting Balb/c mice with 10.sup.7 H. felis. Four weeks after infection, the mice were orally immunized with 200 .mu.g recombinant urease plus 10 .mu.g CT. Control mice were given 10 .mu.g CT only. Antigen was administered 4 times at one week intervals. Animals were sacrificed 4 and 10 weeks after the last immunization, and serum and fecal samples were collected for ELISA.

DOCUMENT-IDENTIFIER: US 5985631 A
TITLE: Method for preventing the activation of inactive, recombinant Helicobacter pylori apourease

Detailed Description Text (6):
Urease polypeptides that can be stabilized using the methods of the invention include urease polypeptides that are purified from Helicobacter (e.g., H. pylori or H. felis) cultures (Michetti et al., WO 94/09823; Dunn et al., J. Biol. Chem. 265:9464-9469; also see below), as well as urease polypeptides that are produced using recombinant methods (e.g., recombinant apourease; Lee et al., J. Infect. Dis. 172:161-172, 1995; Hu et aL, Infect. Immun. 60:2657-2666, 1992; also see below). Though there may be no differences in the amino acid sequences of a native urease and a corresponding recombinant apourease lacking nickel ions, the lack of nickel ions at the active site of the apourease may affect the conformation of the protein, particularly in the active site and nearby regions. Thus, the accessability and reactivity of functional amino acid residues in the active site of the apoprotein is likely to be very different from those of the native protein. The experiments described below show that chemical reagents used for amino acid modification can react with recombinant apourease and influence the activation and stability of the apourease.

Other Reference Publication (9):
Lee et al., "Oral Immunization with Recombinant Helicobacter pylori Urease Induces Secretory IgA Antibodies and Protects Mice from Challenge with Helicobacter felis", J. Inf. Dis., 172:161-172 (1995).

Detailed Description Text (114):
20. Ferrero, R. L. and Lee, A. "The importance of urease in acid protection for the gastric-colonising bacteria Helicobacter pylori and Helicobacter felis sp. nov." Microb. Ecol. Health Dis. 1991, 4, 121-134.

Other Reference Publication (30):
Ferrero and Labigne, Cloning, Expression and Sequencing of Helicobacter felis Urease Genes, Molecular Microbiology 9:323-333, 1993.

Other Reference Publication (38):
Gootz et al., Immunological and Molecular Characterization of Helicobacter felis Urease, Infection and Immunity, 62, 793-798, 1994.

US006258359B1

(12) **United States Patent**
**Labigne et al.**

(10) **Patent No.: US 6,258,359 B1**
(45) **Date of Patent: Jul. 10, 2001**

(54) **IMMUNOGENIC COMPOSITIONS AGAINST HELICOBACTER INFECTION, POLYPEPTIDES FOR USE IN THE COMPOSITIONS, AND NUCLEIC ACID SEQUENCES ENCODING SAID POLYPEPTIDES**

(75) Inventors: **Agnes Labigne**, Bures sur Yvette (FR); **Sebastian Suerbaum**, Veitshöchheim (DE); **Richard L. Ferrero**, Paris; **Jean-Michel Thiberge**, Plaisir, both of (FR)

(73) Assignee: **Institut Pasteur**, Paris (FR)

( * ) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **08/466,248**

(22) Filed: **Jun. 6, 1995**

**Related U.S. Application Data**

(60) Division of application No. 08/447,177, filed on May 19, 1995, now abandoned, which is a continuation-in-part of application No. 08/432,697, filed on May 2, 1995, which is a continuation-in-part of application No. PCT/EP94/01625, filed on May 19, 1994.

(30) **Foreign Application Priority Data**

May 19, 1993 (EP) ..... 93 401 309
Nov. 19, 1993 (WO) ..... PCT/EP93/03259

(51) **Int. Cl.**[7] ..... **A61K 39/395**; A61K 39/40; C07K 1/00; C07K 16/00

(52) **U.S. Cl.** ..... **424/141.1**; 424/150.1; 424/163.1; 424/164.1; 530/350; 530/388.1; 530/388.2; 530/388.4

(58) **Field of Search** ..... 424/234.1, 141.1, 424/150.1, 163.1, 164.1; 514/2; 530/350, 388.1, 388.2, 388.4

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WOA9109049 6/1991 (WO) .
WOA9307273 4/1993 (WO) .
WOA9316723 9/1993 (WO) .
WOA9318150 9/1993 (WO) .
WOA9320843 10/1993 (WO) .
WOA9406474 3/1994 (WO) .
WOA9409823 5/1994 (WO) .
WOA9503824 2/1995 (WO) .
WO 9638475 12/1996 (WO) .
WO 9640893 12/1996 (WO) .

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Michetti et al (Gastroenterology vol. 107 pp 1002–1011), Oct. 1994.*
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R.L. Ferrero et al., "Molecular Evidence Demonstrating Significant Homology Between the Urease Polypeptides of Helicobacter Felis and and Helicobacter Pylori," *Gastroenterology* , vol. 104, No. 4, Apr. 1993, Elsevier, New York, U.S.; P. A699.
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B.E. Dunn et al., "Identification and Purification of a cpn60 Heat Shock Protein Homolog from Helicobacter Pylori, " *Infection and Immunity*, vol. 60, No. 5, May 1992, Am. Soc. Microbiol., Baltimore, US; pp. 1946–1951.
D.J. Evans et al., "Urease–associated Heat Shock Protein of Helicobacter Pylori," *Infection and Immunity*, vol. 60, No. 5, May 1992, Am, Soc. Microbiol., Baltimore, US; pp. 2125–2127.

(List continued on next page.)

*Primary Examiner*—Albert Navarro
(74) *Attorney, Agent, or Firm*—Finnegan, Henderson, Farabow, Garrett and Dunner

(57) **ABSTRACT**

There is provided an immunogenic composition capable of inducing protective antibodies against Helicobacter infection characterized in that it comprises:

i) at least one sub-unit of a urease structural polypeptide from *Helicobacter pylori* (SEQ ID NO: 22,26), or a fragment thereof, said fragment being recognized by antibodies reacting with *Helicobacter felis urease* (SEQ ID NO: 20–21), and/or at least one sub-unit of a urease structural polypeptide from *Helicobacter felis* (SEQ ID NO: 20–21), or a fragment thereof, said fragment being recognized by antibodies reacting with *Helicobacter pylori urease* (SEQ ID NO: 22–26);

ii) and/or, a heat shock protein (Hsp), or chaperonin, from Helicobacter, or a fragment of said protein.

The preparation, by recombinant means, of such immunogenic compositions is also provided.

**20 Claims, 36 Drawing Sheets**

US-PAT-NO: 6258359
DOCUMENT-IDENTIFIER: US 6258359 B1
** See image for Certificate of Correction **

TITLE: Immunogenic compositions against helicobacter infection, polypeptides for use in the compositions, and nucleic acid sequences encoding said polypeptides

DATE-ISSUED: July 10, 2001

INVENTOR-INFORMATION:

| NAME | CITY | STATE | ZIP CODE | COUNTRY |
|---|---|---|---|---|
| Labigne; Agnes | Bures sur Yvette | | | FR |
| Suerbaum; Sebastian | Veitshochheim | | | DE |
| Ferrero; Richard L. | Paris | | | FR |
| Thiberge; Jean-Michel | Plaisir | | | FR |

US-CL-CURRENT: 424/141.1; 424/150.1, 424/163.1, 424/164.1, 530/350, 530/388.1, 530/388.2, 530/388.4

CLAIMS:

What is claimed is:

1. A monoclonal antibody directed against a polypeptide selected from the group consisting of Helicobacter HspA consisting of SEQ ID NO: 29, HspB consisting of SEQ ID NO: 30, and fragments thereof, wherein said fragment is also recognized by an antibody directed against the full length polypeptide corresponding to that fragment.

2. A monoclonal antibody directed against a fragment of HspA of Helicobacter pylori consisting of the following sequence: GSCCHTGNHDHKAKEHEACCHDHKKH (SEQ ID NO: 1).

3. A monoclonal antibody directed against a fragment of HspA of Helicobacter pylori, wherein said HspA of Helicobacter pylori consists of SEQ ID NO: 29, and wherein said fragment has at least 6 consecutive amino acids from the following amino acid sequence:

GSCCHTGNHDHKAKEHEACCHDHKKH (SEQ ID NO: 1) and is recognized by an antibody directed against the full length HspA polypeptide.

4. A composition comprising purified monoclonal antibodies directed against the following peptides:

a) at least one urease polypeptide of Helicobacter felis or Helicobacter pylori selected from the group consisting of UreA, UreB, UreE, UreF, UreG, UreH, UreI, and fragments thereof, wherein said fragment is also recognized by an antibody directed against the full length polypeptide corresponding to that fragment; and

b) at least one polypeptide of Helicobacter felis or Helicobacter pylori selected from the group consisting of HspA (SEQ ID NO: 29), HspB (SEQ ID NO:

30), and fragments thereof, wherein said fragment is also recognized by an antibody directed against the full length polypeptide corresponding to that fragment.

5. The composition of claim 4, wherein said urease polypeptide is UreA of Helicobacter pylori.

6. The composition of claim 4, wherein said urease polypeptide is UreB of Helicobacter pylori.

7. The composition of claim 4, wherein said urease polypeptides are UreA and UreB of Helicobacter pylori.

8. The composition of claim 4, wherein said urease polypeptide is UreA of Helicobacter felis.

9. The composition of claim 4, wherein said urease polypeptide is UreB of Helicobacter felis.

10. The composition of claim 4, wherein said urease polypeptides are UreA and UreB of Helicobacter felis.

11. A method of making an antibody comprising the steps of

providing at least one polypeptide of Helicobacter selected from the group consisting of

(a) Helicobacter heat shock polypeptide HspB (SEQ ID NO: 30),

(b) Helicobacter heat shock polypeptide HspA (SEQ ID NO: 29), and

(c) fragments thereof that are recognized by monoclonal antibodies directed against the full length polypeptide corresponding to that fragment;

immunizing a host animal with said polypeptide; and

purifying said antibody from serum of said host animal.

12. A method of making a monoclonal antibody comprising the steps of

immunizing a host animal with at least one polypeptide of Helicobacter selected from the group consisting of

(a) Helicobacter heat shock polypeptide HspB (SEQ ID NO: 30),

(b) Helicobacter heat shock polypeptide HspA (SEQ ID NO: 29), and

(c) fragments thereof that are recognized by monoclonal antibodies directed against the full length polypeptide corresponding to that fragment;

obtaining a splenocyte from the immunized host animal;

fusing said splenocyte with a myeloma cell;

screening for a hybridoma producing the monoclonal antibody; and

purifying said monoclonal antibody.

13. A method of using an antibody of any one of claims 1, 2, or 3 to induce an immunogenic response in a host animal comprising injecting said antibody into said host animal.

14. A monoclonal antibody directed against a polypeptide encoded by SEQ ID NO: 19.

15. The antibody of claim 14, wherein the polypeptide comprises SEQ ID NO: 20.

16. The antibody of claim 14, wherein the polypeptide comprises SEQ ID NO: 21.

17. A monoclonal antibody directed against an HspA polypeptide encoded by SEQ ID NO: 27.

18. The antibody of claim 17, wherein the polypcptide comprises SEQ ID NO: 29.

19. A purified antibody directed against a UreI polypeptide encoded by SEQ ID NO: 41.

20. The antibody of claim 19, wherein the polypeptide is SEQ ID NO: 42.

US006248330B1

(12) **United States Patent**
**Labigne et al.**

(10) **Patent No.: US 6,248,330 B1**
(45) **Date of Patent: *Jun. 19, 2001**

(54) **IMMUNOGENIC COMPOSITIONS AGAINST HELICOBACTER INFECTION, POLYPEPTIDES FOR USE IN THE COMPOSITIONS, AND NUCLEIC ACID SEQUENCES ENCODING SAID POLYPEPTIDES**

(75) Inventors: **Agnes Labigne**, Bures sur Yvette (FR); **Sebastien Suerbaum**, Bochum (DE); **Richard L. Ferrero**, Paris; **Jean-Michel Thiberge**, Plaisir, both of (FR)

(73) Assignee: **Institut Pasteur**, Paris (FR)

( * ) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **08/432,697**

(22) Filed: **May 2, 1995**

**Related U.S. Application Data**

(63) Continuation-in-part of application No. PCT/EP94/01625, filed on May 19, 1994.

(30) **Foreign Application Priority Data**

May 19, 1993 (EP) ..... 93401309
May 19, 1994 (WO) ..... PCT/EP94/03259

(51) **Int. Cl.**[7] ..... **A61K 39/00**
(52) **U.S. Cl.** ..... **424/192.1**; 424/234.1; 424/184.1; 435/6; 435/69.1
(58) **Field of Search** ..... 424/234.1, 184.1, 424/203.1, 192.1; 435/6, 7.21

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(List continued on next page.)

*Primary Examiner*—Christopher L. Chin
*Assistant Examiner*—Ginny Allen Portner
(74) *Attorney, Agent, or Firm*—Finnegan, Henderson, Farabow, Garrett & Dunner

(57) **ABSTRACT**

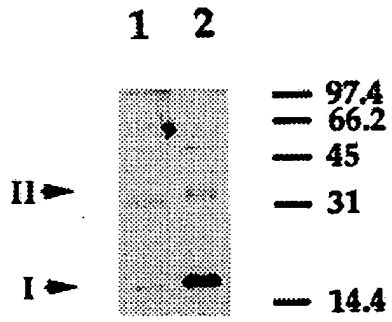
There is provided an immunogenic composition capable of inducing protective antibodies against Helicobacter infection characterized in that it comprises:

i) at least one sub-unit of a urease structural polypeptide from *Helicobacter pylori*, or a fragment thereof, said fragment being recognized by antibodies reacting with *Helicobacter felis* urease, and/or at least one sub-unit of a urease structural polypeptide from *Helicobacter felis*, or a fragment thereof, said fragment being recognized by antibodies reacting with *Helicobacter pylori* urease;

ii) and/or, a heat shock protein (Hsp), or chaperonin, from Helicobacter, or a fragment of said protein.

The preparation, by recombinant means, of such immunogenic compositions is also provided.

**16 Claims, 36 Drawing Sheets**



Brief Summary Text (12):
Indeed, to elucidate the efficiency of individual urease subunits to act as mucosal immunogens, the genes encoding the respective urease sub-units (UreA (SEQ ID NO: 22) and UreB (SEQ ID NO: 21, 26)) of Helicobacter pylori and Helicobacter felis have been cloned in an expression vector (pMAL) and expressed in Escherichia coli cells as translational fusion proteins. The recombinant UreA (SEQ ID NO: 22) and UreB (SEQ ID NO: 21, 26) proteins have been purified by affinity and anion exchange chromatography techniques, and have predicted molecular weights of approximately 68 and 103 kDa, respectively. Western blotting studies indicated that the urease components of the fusion proteins are strongly immunogenic and are specifically recognized by polyclonal rabbit anti-Helicobacter sera. Orogastric immunization of mice with 50 .mu.g of recombinant H. felis UreB (SEQ ID NO: 21), administered in combination with a mucosal adjuvant (cholera toxin), protected 60% ($n=7$; $p<0.005$) of mice from gastric colonization by H. felis bacteria at over 4 months. This compared with a value of 259 ($n=8$; $p>0.05$) for the heterologous H. pylori UreB (SEQ ID NO: 26) antigen. For the first time, a recombinant subunit antigen has been shown to induce an immunoprotective response against gastric Helicobacter infection.

CLAIMS:

10. Proteinaceous material comprising a fusion protein, wherein the fusion protein comprises at least one Helicobacter HspA or a fragment thereof as defined in any one of claims 6-9 and at least one polypeptide selected from the group consisting of

a Helicobacter pylori urease structural polypeptide or fragment thereof, wherein said fragment is recognized by antibodies to H. felis urease, and

a Helicobacter felis urease structural polypeptide or immunogenic fragment thereof.

11. An immunogenic composition, which induces antibodies against Helicobacter infection, comprising at least one sub-unit of a purified, synthetic, or recombinant Helicobacter felis urease structural polypeptide selected from the group of polypeptides consisting of SEQ ID NO: 20 and SEQ ID NO: 21, and a heat shock protein (Hsp) from Helicobacter or a fragment thereof, wherein the Hsp protein is HspA or HspA and HspB encoded by the HspA/HspB genes of plasmid pILL689 (CNCM I-1356), and wherein said fragment has at least 6 amino acids and is immunogenic.

16. An immunogenic composition, capable of inducing antibodies against Helicobacter infection, comprising at least one sub-unit of a purified, synthetic, or recombinant Helicobacter felis urease structural polypeptide selected from the group of polypeptides consisting of SEQ ID NO: 20 and SEQ ID NO: 21, further comprising at least one heat shock protein (Hsp) from Helicobacter, wherein the Hsp protein is HspA, HspB, or HspA and HspB encoded by the HspA/HspB genes of plasmid pILL689 (CNCM I-1356), or a fragment thereof, wherein said fragment has at least 6 amino acids and is capable of generating antibodies.

Detailed Description Text (38):
Particularly preferred antibodies of the invention recognize the Helicobacter felis UreA (SEQ ID NO:20) and/or UreB (SEQ ID NO:21) gene products, that is the A and B urease sub-units. Advantageously, these antibodies also cross-react with the Helicobacter pylori A (SEQ ID NO:22) and B (SEQ ID NO:26) urease sub-units, but do not cross-react with other ureolytic bacteria. Such antibodies may be prepared against epitopes unique to Helicobacter (see FIG. 4), or alternatively, against the whole polypeptides followed by screening out of any antibodies reacting with other ureolytic bacteria.

DOCUMENT-IDENTIFIER: US 6793921 B2
TITLE: Specific antibodies for use in preparation of pharmaceutical compositions useful in the prevention or treatment of gastritis, gastric ulcers and duodenal ulcers

Other Reference Publication (7):
Blanchard et al., "Urease-Specific Monoclonal Antibodies Prevent Helicobacter felis Infection in Mice", Infection and Immunity, 1995, pp. 1394-1399, vol. 63, No. 4, American Society for Microbiology, Washington, D.C.

Other Reference Publication (11):
Ferrero et al., "The Importance of Urease in Acid Protection for the Gastric-colonising Bacteria Helicobacter pylori and Helicobacter felis sp. nov.", Microbial Ecology in Health and Disease, 1991, pp. 121-134, vol. 4, Taylor & Francis, Ltd., Oxfordshire, United Kingdom.

Other Reference Publication (12):
Pappo et al., "Effect of Oral Immunization with Recombinant Urease on Murine Helicobacter felis Gastritis", Infection and Immunity, 1995, pp. 1246-1252, vol. 63, No. 4, American Society for Microbiology, Washington, D.C.

Other Reference Publication (26):
Ermak et al., "Oral Immunization of mice with recombinant Helicobacter pylori urease induces antigen specific IgA+ plasma cells and inhibits colonization of Helicobacter felis in gastric mucosa", FASEB, 1995, p. A216, vol. 9, No. 3, The Federation of American Societies for Experimental Biology, Bethesda, Maryland.

Other Reference Publication (28):
Lee et al., "Oral immunization with recombinant Helicobacter pylori urease induces secretary IgA antibodies and protects mice from challenge with Helicobacter felis", J. Infectious Disease, 1995, pp. 161-172, vol. 172, No. 1, WebMD Medscape Health Network, New York, New York.

Detailed Description Text (6):
Urease polypeptides that can be stabilized using the methods of the invention include urease polypeptides that are purified from Helicobacter (e.g., H. pylori or H. felis) cultures (Michetti et al., WO 94/09823; Dunn et al., J. Biol. Chem. 265:9464-9469; also see below), as well as urease polypeptides that are produced using recombinant methods (e.g., recombinant apourease; Lee et al., J. Infect. Dis. 172:161-172, 1995; Hu et al., Infect. Immun. 60:2657-2666, 1992; also see below). Though there may be no differences in the amino acid sequences of a native urease and a corresponding recombinant apourease lacking nickel ions, the lack of nickel ions at the active site of the apourease may affect the conformation of the protein, particularly in the active site and nearby regions. Thus, the accessability and reactivity of functional amino acid residues in the active site of the apoprotein is likely to be very different from those of the native protein. The experiments described below show that chemical reagents used for amino acid modification can react with recombinant apourease and influence the activation and stability of the apourease.

Other Reference Publication (7):
Lee et al., "Oral Immunization with Recombinant Helicobacter pylori Urease Induces Secretory IgA Antibodies and Protects Mice from Challenge with Helicobacter felis," J. Inf. Dis. 172:161-172 (1995).

41. A vaccine composition consisting essentially of a polypeptide comprising an A subunit of a naturally occurring Helicobacter urease, and a mucosal adjuvant.

42. The vaccine composition of claim 41, wherein said Helicobacter urease is Helicobacter pylori urease.

43. The vaccine composition of claim 41, wherein said Helicobacter urease is Helicobacter felis urease.

44. The vaccine composition of claim 41, wherein said mucosal adjuvant is selected from the group consisting of procholeragenoid; cholera toxin B subunit; fungal polysaccharides, including schizophyllan; muramyl dipeptide; muramyl dipeptide derivatives; phorbol esters; liposomes; microspheres; non-Helicobacter pylori bacterial lysate; labile toxin of Escherichia coli; block polymers; saponins; and ISCOMS.

45. The vaccine composition of claim 41, wherein said polypeptide is genetically or chemically linked to said mucosal adjuvant.

46. The vaccine composition of claim 45, wherein said mucosal adjuvant is cholera toxin B subunit.

47. The vaccine composition of claim 41, further comprising a carrier, such that the composition can be delivered in particulate form.

48. The vaccine composition of claim 47, wherein said carrier is hydroxyapatite.

49. The vaccine composition of claim 41, further comprising a microsphere carrier.

50. The vaccine composition of claim 49, wherein said microsphere carrier is a polylactide-coglycolide biodegradable microsphere carrier.

51. The vaccine composition of claim 41, wherein said composition comprises a recombinant live vector or a recombinant carrier system that expresses said polypeptide.

52. The vaccine composition of claim 51, wherein said live vector is selected from the group consisting of Salmonella typhhimurium, Salmonella typhi, Shigella, Bacillus, Lactobacillus, BCG, Escherichia coli, Vibrio cholerae, Campylobacter, yeast, Herpes virus, Adenovirus, Polio virus, Vaccinia virus, and Avipox.

53. The vaccine composition of claim 51, wherein said carrier system is selected from the group consisting of Bluetongue virus-like particles, Rotaviius virus-like particles, and Ty particles.

54. A vaccine composition comprising a purified polypeptide comprising an A subunit of a naturally occurring Helicobacter urease, and a polypeptide of the labile toxin of Escherichia coli.

55. The vaccine composition of claim 54, wherein said polypeptide of said toxin comprises the B subunit of said toxin.

56. A vaccine composition consisting essentially of a polypeptide comprising a B subunit of a naturally occurring Helicobacter urease, and a mucosal adjuvant.

57. The vaccine composition of claim 56, wherein said Helicobacter urease is Helicobacter pylori urease.

58. The vaccine composition of claim 56, wherein said Helicobacter urease is Helicobacter felis urease.

59. The vaccine composition of claim 56, wherein said mucosal adjuvant is selected from the group consisting of procholeragenoid; cholera toxin B subunit; fungal polysaccharides, including schizophyllan; muramyl dipeptide; muramyl dipeptide derivatives; phorbol esters; liposomes; microspheres; non-Helicobacter pylori bacterial lysate; labile toxin of Escherichia coli; block polymers; saponins; and ISCOMS.

60. The vaccine composition of claim 56, wherein said polypeptide is genetically or chemically linked to said mucosal adjuvant.

61. The vaccine composition of claim 60, wherein said mucosal adjuvant is cholera toxin B subunit.

62. The vaccine composition of claim 56, further comprising a carrier, such that the composition can be delivered in particulate form.

63. The vaccine composition of claim 62, wherein said carnier is hydroxyapatite.

64. The vaccine composition of claim 56, further comprising a microsphere carrier.

65. The vaccine composition of claim 64, wherein said microsphere carrier is a polylactide-coglycolide biodegradable microsphere carrier.

66. The vaccine composition of claim 56, wherein said composition comprises a recombinant live vector or a recombinant carrier system that expresses said polypeptide.

67. The vaccine composition of claim 66, wherein said live vector is selected from the group consisting of Salmonella typhiniurium, Salmonella typhi, Shigella, Bacillus, Lactobacillus, BCG, Escherichia coli, Vibrio cholerae, Campylobacter, yeast, Herpes virus, Adenovirus, Polio virus, Vaccinia virus, and Avipox.

68. The vaccine composition of claim 66, wherein said carrier system is selected from the group consisting of Bluetongue virus-like particles, Rotavirus virus-like particles, and Ty particles.

69. A vaccine composition comprising a purified polypeptide comprising a B subunit of a naturally occurring Helicobacter urease, and a polypeptide of the labile toxin of Escherichia coli.

70. The vaccine composition of claim 69, wherein said polypeptide of said toxin comprises the B subunit of said toxin.

71. A method of preventing Helicobacter infection in a mammal, said method comprising administering to said mammal a prophylactically effective amount of the vaccine composition of claim 54.

72. A method of preventing Helicobacter infection in a mammal, said method comprising administering to said mammal a prop prophylactically effective amount of the vaccine composition of claim 69.

DOCUMENT-IDENTIFIER: US 20020146423 A1
TITLE: PROTECTIVE HELICOBACTER ANTIGENS

Summary of Invention Paragraph:
[0008] The H. felis model has also been used to demonstrate that mice can be orally immunised with Helicobacter antigens, either to protect them from becoming infected (Chen et al, 1992), or to treat them when they are already infected so as to eradicate the infection (Doidge et al, 1994). Antigens that have been used in these vaccines include disrupted cellular preparations from either H. felis or H. pylori, and the bacterial enzyme urease from H. felis or H. pylori or subunits thereof, produced from E. coli clones expressing all or part of the H. pylori urease molecule (Michetti et al, 1994; see also International Patent Publications Nos. WO 90/04030, WO 93/07273 and WO 94/09823). H. pylori heat shock protein (Hsp or HSP) has also been shown to be a protective antigen (Ferrero et al., 1995).

Summary of Invention Paragraph:
[0009] International Patent Publication No. WO 93/18150 (Application No. PCT/EP93/00472) discloses vaccines or therapeutic compositions comprising one or more of recombinant H. pylori cytotoxin (CT or VacA), H. pylori cytotoxin-associated immunodominant antigen (CAI or CagA) or H. pylori heat shock protein, optionally together with H. pylori urease. International Patent Publication No. WO 95/27506 (Application No. PCT/FR95/00383) discloses an anti-H. pylori immunising composition containing a substantially purified H. pylori catalase as the active ingredient; and International Patent Publication No. WO 95/14093 (Application No. PCT/EP93/03259) discloses an immunogenic composition capable of inducing protective antibodies against Helicobacter infection which comprises at least one urease structural polypeptide from H. pylori or H. felis and optionally a urease-associated heat shock protein or chaperonin from Helicobacter.

Detail Description Paragraph:
[0247] These results show that ora-gastric immunisation with any of the five purified recombinant proteins in conjunction with a mucosal adjuvant protected mice from infection with H. pylori. The results of the unpurified E. coli whole-cell sonicates suggest that higher levels of expression or purification are required to demonstrate protection. The gene screening strategy, using serum from immune mice (immunised with H. felis sonicate), identified two known H. pylori protective antigens, urease and heat shock protein, and five other proteins. The results reported here now show that these five are also protective antigens. One of the five antigens is a previously known compound (Kostrzynska et al, 1994), but it was not previously known whether this compound was a protective antigen. As we have shown that protective immunogenic preparations can be used to treat infection, as well as prevent it, it would be expected that these protective antigens could be used to treat, as well as prevent, Helicobacter infection in humans. The validity of the Helicobacter felis mouse model, that was used to identify these Helicobacter pylori antigens, has been shown by the ability of these antigens to protect mice in a recently developed H. pylori mouse model. It would therefore be expected, that these antigens, alone or in combination, would be protective antigens in products used to treat or prevent Helicobacter infections in humans.

Detail Description Paragraph:
[0259] Michetti, P., Corth'sy-Theulaz, I., Davin, C., Haas, R., Vaney, A-C., Heitz, M., Bille, J., Kraehenbuhl, J-P., Saraga, E. and Blum, A. L. (1994). Immunization of BALB/c mice against Helicobacter felis infection with Helicobacter pylori urease. Gastroenterology 107:1002-1011.

PGPUB-DOCUMENT-NUMBER: 20030007980
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20030007980 A1

TITLE: Urease-based vaccine and treatment for helicobacter infection

PUBLICATION-DATE: January 9, 2003

INVENTOR-INFORMATION:

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APPL-NO: 09/955739 [PALM]
DATE FILED: September 18, 2001

RELATED-US-APPL-DATA:
Application 09/955739 is a continuation-of US application 08/200346, filed February 23, 1994, US Patent No. 6290962
Application 08/200346 is a continuation-in-part-of US application 08/085938, filed July 6, 1993, US Patent No. 5972336
Application 08/085938 is a continuation-in-part-of US application 07/970996, filed November 3, 1992, ABANDONED

INT-CL-PUBLISHED: [07] A61K 39/02

INT-CL-CURRENT:

| TYPE | IPC | DATE |
|---|---|---|
| CIPS | C12 N 9/78 | 20060101 |
| CIPS | C12 N 9/80 | 20060101 |

US-CL-PUBLISHED: 424/190.1; 424/234.1
US-CL-CURRENT: 424/190.1; 424/234.1

REPRESENTATIVE-FIGURES: NONE

ABSTRACT:

Method of eliciting in a mammalian host a protective immune response to Helicobacter infection and treatment of Helicobacter infection by administering to the host an immunogenically effective amount of

a Helicobacter urease or urease subunits as antigen. Vaccine compositions are also provided.

RELATED APPLICATIONS

[0001] This application is a continuation-in-part of U.S. application Ser. No. 08/085,938, filed Jul. 6, 1993, which is a continuation-in-part application of U.S. application Ser. No. 07/970,996, filed Nov. 3, 1992, the whole of which applications (including drawings) are hereby incorporated by reference.

44. A composition useful in the therapeutic treatment of gastroduodenal disease, said composition comprising Helicobacter urease peptides.

45. The composition of claim 44 wherein said gastroduodenal disease is gastritis.

46. The composition of claim 44 wherein said gastroduodenal disease is peptic ulcer disease.

47. The composition of claim 44 wherein said gastroduodenal disease is chronic dyspepsia with severe erosive gastroduodenitis.

48. The composition of claim 44 wherein said gastroduodenal disease is refractory non-ulcer dyspepsia.

49. The composition of claim 44 wherein said gastroduodenal disease is intestinal metaplasia.

50. The composition of claim 44 wherein said gastroduodenal disease is low grade MALT lymphoma.

51. The composition of claim 44 wherein said gastroduodenal disease is Helicobacter infection.

52. The composition of claim 44 wherein said gastroduodenal disease is Helicobacter pylori infection.

53. The composition of claim 44 wherein said gastroduodenal disease is Helicobacter felis disease.

54. The composition of claim 44 wherein said mammal is human.

55. The composition of claim 44 wherein said Helicobacter urease peptides comprise Helicobacter urease.

56. The composition of claim 44 wherein said Helicobacter urease peptides comprise the ureB subunit of Helicobacter urease.

57. The composition of claim 44 wherein said Helicobacter urease peptides comprise Helicobacter pylori urease.

58. The composition of claim 44 wherein said Helicobacter urease comprise the ure B subunit of Helicobacter pylori urease.

59. The composition of claim 44 further comprising a mucosal adjuvant.

60. The composition of claim 59 wherein said mucosal adjuvant is selected from the group consisting of procholeragenoid; cholera toxin B subunit fungal polysaccharides, including schizophyllan; muramyl dipeptide; muramyl dipeptide derivatives; phorbol esters; liposomes; microspheres; non-Helicobacter pylori bacterial lysates; labile toxin of Escherichia coli; block polymers; saponins; and ISCOMs.

61. The composition of claim 44 wherein said urease peptides are genetically or chemically linked to a mucosal adjuvant.

62. The composition of claim 61, wherein said mucosal adjuvant is cholera toxin B subunit.

63. The composition of claim 44 further comprising a carrier such that the composition can be delivered in particulate form.

64. The composition of claim 44 wherein said carrier is hydroxyapatite.

65. The composition of claim 44 further comprising a microsphere carrier.

66. The composition of claim 65, wherein said microsphere carrier is a polylactide-coglycolide biodegradable microsphere carrier.

67. The composition of claim 44 wherein said composition comprises a recombinant live vector or a recombinant carrier system which expresses a Helicobacter urease peptide.

68. The composition of claim 67 wherein said live vector is selected from the group consisting of Salmonella typhimurium, Salmonella typhi, Shigella, Bacillus, Lactobacillus, BCG, Escherichia coli, Vibrio cholerae, Campylobacter, Yeast, Herpes virus, Adenovirus, Poliovirus, Vaccinia, and Avipox.

69. The composition of claim 67 wherein said carrier system is selected from the group consisting of Bluetongue virus-like particles, Rotavirus virus-like particles, and Ty particles.

70. A composition useful in the therapeutic treatment of Helicobacter pylori infection of a human, said composition comprising the ure B subunit of Helicobacter pylori urease, a mucosal adjuvant selected from a group consisting of procholeragenoid; cholera toxin B subunit fungal polysaccharides, including schizophyllan; muramyl dipeptide; muramyl dipeptide derivatives; phorbol esters; liposomes; microspheres; non-Helicobacter pylori bacterial lysates; labile toxin of Escherichia coli; block polymers; saponins; and ISCOMs, and further comprising hydroxyapatite.

71. A composition useful in the therapeutic treatment of Helicobacter pylori infection of a human, said composition comprising the ure B subunit of Helicobacter pylori urease in the form of a fused protein, genetically linked to the cholera toxin B subunit and hydroxyapatite, in particulate form.

72. A composition useful for the therapeutic treatment of a mammal infected with Helicobacter, said composition comprising peptides that display epitopes sufficiently homologous to epitopes displayed by Helicobacter urease such that antibodies that recognize epitopes displayed by Helicobacter urease will recognize epitopes displayed by said peptides.

73. A composition useful for the therapeutic treatment of a mammal infected with Helicobacter said composition comprising anti-idiotypic antibodies to Helicobacter urease.

74. A composition useful in the therapeutic treatment of gastroduodenal disease, said composition comprising an antibody that recognizes Helicobacter urease.

75. The composition of claim 74 wherein said gastroduodenal disease is Helicobacter infection.

76. The composition of claim 74 wherein said gastroduodenal disease is Helicobacter pylori infection.

77. The composition of claim 74 wherein said gastroduodenal disease is Helicobacter felis disease.

78. The composition of claim 74 wherein said mammal is human.

79. The composition of claim 74 wherein the antibody is specific for Helicobacter pylori urease.

80. The composition of claim 74 wherein the antibody is specific for the ure B subunit of Helicobacter

pylori urease.

81. The composition of claim 74 wherein the antibody is a monoclonal antibody.

82. The composition of claim 74 wherein the antibody is an IgA antibody.

83. A composition useful in the therapeutic treatment of gastroduodenal disease, said composition comprising an IgA monoclonal antibody that recognizes the ure B subunit of Helicobacter pylori urease and a mucosal adjuvant.

84. A method of preventing Helicobacter pylori infection of a human, said method comprising orally administering a pro-phylactically effective amount of a composition comprising Helicobacter pylori urease, in association with a mucosal adjuvant selected from the group consisting of procholeragenoid; cholera toxin B subunit; fungal saccharides, including schizophyllan; muramyl dipeptide; muramyl dipeptide derivatives; phorbol esters; liposomes; microspheres; non-Helicobacter pylori bacterial lysates; labile toxin of Escherichia coli; block polymers; saponins; and ISCOMs, said composition administered in particulate form in association with hydroxyapatite.

85. A method of preventing Helicobacter pylori infection of a human, said method comprising orally administering a prophylactically effective amount of a composition comprising Helicobacter pylori urease, in association with a mucosal adjuvant selected from the group consisting of procholeragenoid; cholera toxin B subunit; fungal saccharides, including schizophyllan; muramyl dipeptide; muramyl dipeptide derivatives; phorbol esters; liposomes; microspheres; non-Helicobacter pylori bacterial lysates; labile toxin of Escherichia coli; block polymers; saponins; and ISCOMs, said composition administered in particulate form in association with hydroxyapatite.

86. A method of preventing Helicobacter pylori infection of a human, said method comprising orally administering a pro-phylactically effective amount of a composition comprising Helicobacter pylori urease in the form of a fused protein, genetically linked to the cholera toxin B subunit, said composition administered in particulate form, in association with hydroxyapatite.

87. A composition useful in preventing Helicobacter pylori infection of a human, said composition comprising Helicobacter pylori urease, in association with a mucosal adjuvant selected from the group consisting of procholeragenoid; cholera toxin B subunit; fungal polysaccharides, including schizophyllan; muramyl dipeptide; muramyl dipeptide derivatives; phorbol esters; liposomes; microspheres; non-Helicobacter pylori bacterial lysates; labile toxin of Escherichia coli; block polymers; saponins; and ISCOMs, said composition present in particulate form in association with hydroxyapatite.

88. A composition useful in preventing Helicobacter pylori infection of a human, said composition comprising Helicobacter pylori urease in the form of a fused protein, genetically linked to the cholera toxin B subunit, said fused protein present in particulate form, in association with hydroxyapatite.

Detail Description Paragraph:
[0063] For example, although the amino acid sequences of the UreA and UreB subunits of H. pylori and H. felis ureases differ from one another by 26.5% and 11.8%, respectively (Ferrero et al., Molecular Microbiology 9(2):323-333, 1993), it has been shown that H. pylori urease protects mice from H. felis infection (Michetti et al., Gastroenterology 107:1002-1011, 1994). In addition, it has been shown that the individual structural subunits of urease, UreA and UreB, which contain distinct amino acid sequences, are both protective antigens against Helicobacter infection (Michetti et al., supra). Similarly, Cuenca et al. (Gastroenterology 110: 1770-1775, 1996) showed that therapeutic immunization of H. mustelae-infected ferrets with H. pylori urease was effective at eradicating H. mustelae infection. Further, several urease variants have been reported to be effective vaccine antigens, including, e.g., recombinant UreA+UreB apoenzyme expressed from pORV142 (UreA and UreB sequences derived from H. pylori strain CPM630; Lee et al., J. Infect. Dis. 172:161-172, 1995); recombinant UreA+UreB apoenzyme expressed from pORV214 (UreA and UreB sequences differ from H. pylori strain CPM630 by one and two amino acid changes, respectively; Lee et al., supra, 1995); a UreA-glutathione-S-transferase fusion protein (UreA sequence from H. pylori strain ATCC 43504; Thomas et al., Acta Gastro-Enterologica Belgica, 56:54, September 1993); UreA+UreB holoenzyme purified from H. pylori strain NCTC11637 (Marchetti et al., Science 267:1655-1658, 1995); a UreA-MBP fusion protein (UreA from H. pylori strain 85P; Ferrero et al., Infection and Immunity 62:4981-4989, 1994); a UreB-MBP fusion protein (UreB from H. pylori strain 85P; Ferrero et al., supra); a UreA-MBP fusion protein (UreA from H. felis strain ATCC 49179; Ferrero et al., supra); a UreB-MBP fusion protein (UreB from H. felis strain ATCC 49179; Ferrero et al., supra); and a 37 kD fragment of UreB containing amino acids 220-569 (Dore-Davin et al., "A 37 kD fragment of UreB is sufficient to confer protection against Helicobacter felis infection in mice"). Finally, Thomas et al. (supra) showed that oral immunization of mice with crude sonicates of H. pylori protected mice from subsequent challenge with H. felis.

Detail Description Paragraph:
[0177] Therapeutic or prophylactic efficacy can be evaluated using standard methods in the art, e.g., by measuring induction of a mucosal immune response or induction of protective and/or therapeutic immunity, using, e.g., the H. felis mouse model and the procedures described in Lee et al. (Eur. J. Gastroenterology and Hepatology 7:303, 1995) or Lee et al. (J. Infect. Dis. 172:161, 1995). Those skilled in the art will recognize that the H. felis strain of the model can be replaced with another Helicobacter strain. For example, the efficacy of DNA molecules and polypeptides from H. pylori is preferably evaluated in a mouse model using an H. pylori strain. Protection can be determined by comparing the degree of Helicobacter infection in the gastric tissue (assessed by urease activity, bacterial counts or gastritis) to that of a control group. Protection is shown when infection is reduced by comparison to the control group. Such an evaluation can be made for polynucleotides, vaccine vectors, polypeptides and derivatives thereof, as well as antibodies of the invention.

US 20030158396A1

(19) United States

(12) Patent Application Publication (10) Pub. No.: US 2003/0158396 A1

Kleanthous et al. (43) Pub. Date: Aug. 21, 2003

(54) IDENTIFICATION OF POLYNUCLEOTIDES ENCODING NOVEL HELICOBACTER POLYPEPTIDES IN THE HELICOBACTER GENOME

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(21) Appl. No.: **09/882,227**

(22) Filed: **Jun. 15, 2001**

**Related U.S. Application Data**

(63) Continuation of application No. 08/902,615, filed on Jul. 29, 1997, now abandoned.

**Publication Classification**

(51) **Int. Cl.**[7] ..... **C07H 21/02**; C07H 21/04

(52) **U.S. Cl.** ..... **536/23.1**

(57) **ABSTRACT**

The invention provides Helicobacter polypeptides that can be used in vaccination methods for preventing or treating Helicobacter infection, and polynucleotides that encode these polypeptides.

DOCUMENT-IDENTIFIER: US 20030158396 A1
TITLE: Identification of polynucleotides encoding novel helicobacter polypeptides in the helicobacter genome

Summary of Invention Paragraph:
[0024] For example, although the amino acid sequences of the UreA and UreB subunits of H. pylori and H. felis ureases differ from one another by 26.5% and 11.8%, respectively (Ferrero et al., Molecular Microbiology 9(2):323-333, 1993), it has been shown that H. pylori urease protects mice from H. felis infection (Michetti et al., Gastroenterology 107:1002, 1994). In addition, it has been shown that the individual structural subunits of urease, UreA and UreB, which contain distinct amino acid sequences, are both protective antigens against Helicobacter infection (Michetti et al., supra). Similarly, Cuenca et al. (Gastroenterology 110:1770, 1996) showed that therapeutic immunization of H. mustelae-infected ferrets with H. pylori urease was effective at eradicating H. mustelae infection. Further, several urease variants have been reported to be effective vaccine antigens, including, e.g., recombinant UreA+UreB apoenzyme expressed from pORV142 (UreA and UreB sequences derived from H. pylori strain CPM630; Lee et al., J. Infect. Dis.172:161, 1995); recombinant UreA+UreB apoenzyme expressed from pORV214 (UreA and UreB sequences differ from H. pylori strain CPM630 by one and two amino acid changes, respectively; Lee et al., supra, 1995); a UreA-glutathione-S-transferase fusion protein (UreA sequence from H. pylori strain ATCC 43504; Thomas et al., Acta Gastro-Enterologica Belgica 56:54, 1993); UreA+UreB holoenzyme purified from H. pylori strain NCTC11637 (Marchetti et al., Science 267:1655, 1995); a UreA-MBP fusion protein (UreA from H. pylori strain 85P; Ferrero et al., Infection and Immunity 62:4981, 1994); a UreB-MBP fusion protein (UreB from H. pylori strain 85P; Ferrero et al., supra); a UreA-MBP fusion protein (UreA from H. felis strain ATCC 49179; Ferrero et al., supra); a UreB-MBP fusion protein (UreB from H. felis strain ATCC 49179; Ferrero et al., supra); and a 37 kDa fragment of UreB containing amino acids 220-569 (Dore-Davin et al., "A 37 kD fragment of UreB is sufficient to confer protection against Helicobacter felis infection in mice"). Finally, Thomas et al. (supra) showed that oral immunization of mice with crude sonicates of H. pylori protected mice from subsequent challenge with H. felis.

Summary of Invention Paragraph:
[0094] Therapeutic or prophylactic efficacy can be evaluated using standard methods in the art, e.g., by measuring induction of a mucosal immune response or induction of protective and/or therapeutic immunity, using, e.g., the H. felis mouse model and the procedures described by Lee et al. (Eur. J. Gastroenterology & Hepatology 7:303, 1995) or Lee et al. (J. Infect. Dis. 172:161, 1995). Those skilled in the art will recognize that the H. felis strain of the model can be replaced with another Helicobacter strain. For example, the efficacy of polynucleotide molecules and polypeptides from H. pylori is, preferably, evaluated in a mouse model using an H. pylori strain. Protection can be determined by comparing the degree of Helicobacter infection in the gastric tissue assessed by, for example, urease activity, bacterial counts, or gastritis, to that of a control group. Protection is shown when infection is reduced by comparison to the control group. Such an evaluation can be made for polynucleotides, vaccine vectors, polypeptides, and polypeptide derivatives, as well as for antibodies of the invention.

PGPUB-DOCUMENT-NUMBER: 20030158396
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20030158396 A1

TITLE: Identification of polynucleotides encoding novel helicobacter polypeptides in the helicobacter genome

PUBLICATION-DATE: August 21, 2003

INVENTOR-INFORMATION:

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APPL-NO: 09/882227 [PALM]
DATE FILED: June 15, 2001

RELATED-US-APPL-DATA:
Application 09/882227 is a continuation-of US application 08/902615, filed July 29, 1997, ABANDONED

INT-CL-PUBLISHED: [07] C07H 21/02, C07H 21/04

INT-CL-CURRENT:

| TYPE | IPC | DATE |
|---|---|---|
| CIPS | C07 K 14/195 | 20060101 |
| CIPS | C07 K 14/205 | 20060101 |
| CIPN | A61 K 38/00 | 20060101 |
| CIPN | A61 K 39/00 | 20060101 |
| CIPS | C07 K 16/12 | 20060101 |

US-CL-PUBLISHED: 536/23.1
US-CL-CURRENT: 536/23.1

ABSTRACT:

The invention provides Helicobacter polypeptides that can be used in vaccination methods for preventing or treating Helicobacter infection, and polynucleotides that encode these polypeptides.

PRIORITY INFORMATION

[0001] This application is a continuation of, and claims priority from, U.S. Ser. No. 08/902,615, filed on

Jul. 29, 1997, which is incorporated by reference herein in its entirety.

[0156] 4.C. Adsorption and Elution of the Antigen

[0157] An antigen solution in 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, for example, the supernatant or the solubilized pellet obtained using the methods described in 3.E., after centrifugation and filtration through a 0.45 μm membrane, is applied to a column equilibrated with 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, at a flow rate of about 10 ml/hour. The column is then washed with 20 volumes of 50 mM Tris-HCl (pH 8.0), 2 mM EDTA. Alternatively, adsorption can be achieved by mixing overnight at 5±3° C.

[0158] The adsorbed gel is washed with 2 to 6 volumes of 10 mM sodium phosphate buffer (pH 6.8) and the antigen is eluted with 100 mM glycine buffer (pH 2.5). The eluate is recovered in 3 ml fractions, to each of which is added 150 μl of 1 M sodium phosphate buffer (pH 8.0). Absorption is measured at 280 nm for each fraction; those fractions containing the antigen are pooled and stored at −20° C.

[0159] Other embodiments are within the following claims.

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SEQUENCE LISTING

The patent application contains a lengthy "Sequence Listing" section. A copy of the "Sequence Listing" is available in electronic form from the USPTO web site (http://seqdata.uspto.gov/sequence.html?DocID=20030158396). An electronic copy of the "Sequence Listing" will also be available from the USPTO upon request and payment of the fee set forth in 37 CFR 1.19(b)(3).

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What is claimed is:

1. An isolated polynucleotide that encodes:

(i) a polypeptide comprising an amino acid sequence that is homologous to the amino acid sequence of a Helicobacter polypeptide selected from the group consisting of GHPO7 (SEQ ID NO: 2), GHPO8 (SEQ ID NO: 4), GHPO9 (SEQ ID NO: 6), GHPO10 (SEQ ID NO: 8), GHPO12 (SEQ ID NO: 10), GHPO25 (SEQ ID NO: 12), GHPO27 (SEQ ID NO: 14), GHPO29 (SEQ ID NO: 16), GHPO30 (SEQ ID NO: 18), GHPO37 (SEQ ID NO: 20), GHPO49 (SEQ ID NO: 22), GHPO51 (SEQ ID NO: 24), GHPO54 (SEQ ID NO: 26), GHPO65 (SEQ ID NO: 28), GHPO66 (SEQ ID NO: 30), GHPO68 (SEQ ID NO: 32), GHPO70 (SEQ ID NO: 34), GHPO77 (SEQ ID NO: 36), GHPO83 (SEQ ID NO: 38), GHPO85 (SEQ ID NO: 40), GHPO87 (SEQ ID NO: 42), GHPO91 (SEQ ID NO: 44), GHPO92 (SEQ ID NO: 46), GHPO96 (SEQ ID NO: 48), GHPO97 (SEQ ID NO: 50), GHPO111 (SEQ ID NO: 52), GHPO115 (SEQ ID NO: 54), GHPO117 (SEQ ID NO: 56), GHPO123 (SEQ ID NO: 58), GHPO124 (SEQ ID NO: 60), GHPO126 (SEQ ID NO: 62), GHPO127 (SEQ ID NO: 64), GHPO128 (SEQ ID NO: 66), GHPO131 (SEQ ID NO: 68), GHPO133 (SEQ ID NO: 70), GHPO140 (SEQ ID NO: 72), GHPO141 (SEQ ID NO: 74), GHPO145 (SEQ ID NO: 76), GHPO147 (SEQ ID NO: 78), GHPO166 (SEQ ID NO: 80), GHPO181 (SEQ ID NO: 82), GHPO187 (SEQ ID NO: 84), GHPO188 (SEQ ID NO: 86), GHPO192 (SEQ ID NO: 88), GHPO202 (SEQ ID NO: 90), GHPO204 (SEQ ID NO: 92), GHPO205 (SEQ ID NO: 94), GHPO212 (SEQ ID NO: 96), GHPO218 (SEQ ID NO: 98), GHPO226 (SEQ ID NO: 100), GHPO231 (SEQ ID NO: 102), GHPO236 (SEQ ID NO: 104), GHPO239 (SEQ ID NO: 106), GHPO245 (SEQ ID NO: 108), GHPO246 (SEQ ID NO: 110), GHPO248 (SEQ ID NO: 112), GHPO253 (SEQ ID NO: 114), GHPO265 (SEQ ID NO: 116), GHPO266 (SEQ ID NO: 118), GHPO271 (SEQ ID NO: 120), GHPO272 (SEQ ID NO: 122), GHPO286 (SEQ ID NO: 124), GHPO291 (SEQ ID NO: 126), GHPO292 (SEQ ID NO: 128), GHPO297 (SEQ ID NO: 130), GHPO304 (SEQ ID NO: 132), GHPO307 (SEQ ID NO: 134), GHPO324 (SEQ ID NO: 136), GHPO326 (SEQ ID NO: 138), GHPO331 (SEQ ID NO: 140), GHPO343 (SEQ ID NO: 142), GHPO345 (SEQ ID NO: 144), GHPO346 (SEQ ID NO: 146), GHPO352 (SEQ ID NO: 148), GHPO355 (SEQ ID NO: 150), GHPO363 (SEQ ID NO: 152), GHPO369 (SEQ ID NO: 154), GHPO376 (SEQ ID NO: 156), GHPO378 (SEQ ID NO: 158), GHPO388 (SEQ ID NO: 160), GHPO396 (SEQ ID NO: 162), GHPO403 (SEQ ID NO: 164), GHPO410 (SEQ ID NO: 166), GHPO415 (SEQ ID NO: 168), GHPO421 (SEQ ID NO: 170), GHPO439 (SEQ ID NO: 172), GHPO441 (SEQ ID NO: 174), GHPO443 (SEQ ID NO: 176), GHPO453 (SEQ ID NO: 178), GHPO455 (SEQ ID NO: 180), GHPO464 (SEQ ID NO: 182), GHPO467 (SEQ ID NO: 184), GHPO468 (SEQ ID NO: 186), GHPO470 (SEQ ID NO: 188), GHPO486 (SEQ ID NO: 190), GHPO487 (SEQ ID NO: 192), GHPO488 (SEQ ID NO: 194), GHPO489 (SEQ ID NO: 196), GHPO498 (SEQ ID NO: 198), GHPO501 (SEQ ID NO: 200), GHPO504 (SEQ ID NO: 202), GHPO512 (SEQ ID NO: 204), GHPO517 (SEQ ID NO: 206), GHPO520 (SEQ ID NO: 208), GHPO528 (SEQ ID NO: 210), GHPO530 (SEQ ID NO: 212), GHPO532 (SEQ ID NO: 214), GHPO548 (SEQ ID NO: 216), GHPO561 (SEQ ID NO: 218), GHPO564 (SEQ ID NO: 220), GHPO572 (SEQ ID NO: 222), GHPO573 (SEQ ID NO: 224), GHPO574 (SEQ ID NO: 226), GHPO577 (SEQ ID NO: 228), GHPO579 (SEQ ID NO: 230), GHPO583 (SEQ ID NO: 232), GHPO588 (SEQ ID NO: 234), GHPO593 (SEQ ID NO: 236), GHPO597 (SEQ ID NO: 238), GHPO598 (SEQ ID NO: 240), GHPO604 (SEQ ID NO: 242), GHPO606 (SEQ ID NO: 244), GHPO611 (SEQ ID NO: 246), GHPO612 (SEQ ID NO: 248), GHPO615 (SEQ ID NO: 250), GHPO632 (SEQ ID NO: 252), GHPO633 (SEQ ID NO: 254), GHPO637 (SEQ ID NO: 256), GHPO651

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(ii) a derivative of said Helicobacter polypeptide.

2. The isolated polynucleotide of claim 1, which encodes a mature form of said Helicobacter polypeptide.

3. The isolated polynucleotide of claim 1, wherein the polynucleotide is a DNA molecule.

4. The isolated polynucleotide of claim 1, which is a DNA molecule that can be amplified by polymerase chain reaction from a Helicobacter genome.

5. The isolated DNA molecule of claim 4, which can be amplified by the polymerase chain reaction from a *Helicobacter pylori* genome.

6. The isolated polynucleotide of claim 1, which is a DNA molecule that encodes the mature form or a derivative of a polypeptide encoded by the DNA molecule of claim 4.

7. The isolated polynucleotide of claim 1, which is a DNA molecule that encodes the mature form or a derivative of a polypeptide encoded by the DNA molecule of claim 5.

8. A compound, in a substantially purified form, that is the mature form or a derivative of a polypeptide comprising an amino acid sequence that is homologous to a Helicobacter polypeptide selected from the group consisting of GHPO7 (SEQ ID NO: 2), GHPO8 (SEQ ID NO: 4), GHPO9 (SEQ ID NO: 6), GHPO10 (SEQ ID NO: 8), GHPO12 (SEQ ID NO: 10), GHPO25 (SEQ ID NO: 12), GHPO27 (SEQ ID NO: 14), GHPO29 (SEQ ID NO: 16), GHPO30 (SEQ ID NO: 18), GHPO37 (SEQ ID NO: 20), GHPO49 (SEQ ID NO: 22), GHPO51 (SEQ ID NO: 24), GHPO54 (SEQ ID NO: 26), GHPO65 (SEQ ID NO: 28), GHPO66 (SEQ ID NO: 30), GHPO68 (SEQ ID NO: 32), GHPO70 (SEQ ID NO: 34), GHPO77 (SEQ ID NO: 36), GHPO83 (SEQ ID NO: 38), GHPO85 (SEQ ID NO: 40), GHPO87 (SEQ ID NO: 42), GHPO91 (SEQ ID NO: 44), GHPO92 (SEQ ID NO: 46), GHPO96 (SEQ ID NO: 48), GHPO97 (SEQ ID NO: 50), GHPO111 (SEQ ID NO: 52), GHPO115 (SEQ ID NO: 54), GHPO117 (SEQ ID NO: 56), GHPO123 (SEQ ID NO: 58), GHPO124 (SEQ ID NO: 60), GHPO126 (SEQ ID NO: 62), GHPO127 (SEQ ID NO: 64), GHPO128 (SEQ ID NO: 66), GHPO131 (SEQ ID NO: 68), GHPO133 (SEQ ID NO: 70), GHPO140 (SEQ ID NO: 72), GHPO141 (SEQ ID NO: 74), GHPO145 (SEQ ID NO: 76), GHPO147 (SEQ ID NO: 78), GHPO166 (SEQ ID NO: 80), GHPO181 (SEQ ID NO: 82), GHPO187 (SEQ ID NO: 84), GHPO188 (SEQ ID NO: 86), GHPO192 (SEQ ID NO: 88), GHPO202 (SEQ ID NO: 90), GHPO204 (SEQ ID NO: 92), GHPO205 (SEQ ID NO: 94), GHPO212 (SEQ ID NO: 96), GHPO218 (SEQ ID NO: 98), GHPO226 (SEQ ID NO: 100), GHPO231 (SEQ ID NO: 102), GHPO236 (SEQ ID NO: 104), GHPO239 (SEQ ID NO: 106), GHPO245 (SEQ ID NO: 108), GHPO246 (SEQ ID NO: 110), GHPO248 (SEQ ID NO: 112), GHPO253 (SEQ ID NO: 114), GHPO265 (SEQ ID NO: 116), GHPO266 (SEQ ID NO: 118), GHPO271 (SEQ ID NO: 120), GHPO272 (SEQ ID NO: 122), GHPO286 (SEQ ID NO: 124), GHPO291 (SEQ ID NO: 126), GHPO292 (SEQ ID NO: 128), GHPO297 (SEQ ID NO: 130), GHPO304 (SEQ ID NO: 132), GHPO307 (SEQ ID NO: 134), GHPO324 (SEQ ID NO: 136), GHPO326 (SEQ ID NO: 138), GHPO331 (SEQ ID NO: 140), GHPO343 (SEQ ID NO: 142), GHPO345 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(ii) a derivative of said Helicobacter polypeptide.

**9**. The compound of claim 8, which is the mature form or a derivative of a polypeptide encoded by a DNA molecule of claim 4.

**10**. The compound of claim 8, which is the mature form or a derivative of a polypeptide encoded by a DNA molecule of claim 5.

**11**. A method of preventing or treating Helicobacter infection in a mammal, said method comprising administering to said mammal a prophylactically or therapeutically effective amount of a compound of claim 8.

**12**. The method of claim 11, further comprising administering to said mammal an antibiotic, an antisecretory agent, a bismuth salt, or a combination thereof.

**13**. The method of claim 12, wherein said antibiotic is selected from the group consisting of amoxicillin, clarithromycin, tetracycline, metronidizole, and erythromycin.

**14**. The method of claim 12, wherein said bismuth salt is selected from the group consisting of bismuth subcitrate and bismuth subsalicylate.

**15**. The method of claim 12, wherein said antisecretory agent is a proton pump inhibitor.

**16**. The method of claim 15, wherein said proton pump inhibitor is selected from the group consisting of omeprazole, lansoprazole, and pantoprazole.

**17**. The method of claim 12, wherein said antisecretory agent is an $H_2$-receptor antagonist.

**18**. The method of claim 17, wherein said $H_2$-receptor antagonist is selected from the group consisting of ranitidine, cimetidine, famotidine, nizatidine, and roxatidine.

**19**. The method of claim 12, wherein said antisecretory agent is a prostaglandin analog.

**20**. The method of claim 19, wherein said prostaglandin analog is misoprostil or enprostil.

**21**. The method of claim 11, further comprising administering to said mammal a prophylactically or therapeutically effective amount of a second Helicobacter polypeptide or a derivative thereof.

**22**. The method of claim 21, wherein the second Helicobacter polypeptide is a Helicobacter urease, or a subunit or a derivative thereof.

**23**. A composition comprising a compound of claim 8, together with a physiologically acceptable diluent or carrier.

**24**. The composition of claim 23, further comprising an adjuvant.

**25**. The composition of claim 23, further comprising a second Helicobacter polypeptide or a derivative thereof.

**26**. The composition of claim 25, wherein said second Helicobacter polypeptide is a Helicobacter urease, or a subunit or a derivative thereof.

**27**. A method of preventing or treating Helicobacter infection in a mammal, said method comprising administering to said mammal a prophylactically or therapeutically effective amount of a polynucleotide of claim 1.

**28**. A method of preventing or treating Helicobacter infection in a mammal, said method comprising administering to said mammal a prophylactically or therapeutically effective amount of a polynucleotide of claim 4.

**29**. A method of preventing or treating Helicobacter infection in a mammal, said method comprising administering to said mammal a prophylactically or therapeutically effective amount of a polynucleotide of claim 7.

**30**. A composition comprising a viral vector, in the genome of which is inserted a DNA molecule of claim 3, said DNA molecule being placed under conditions for expression in a mammalian cell and said viral vector being admixed with a physiologically acceptable diluent or carrier.

**31**. The composition of claim 30, wherein said viral vector is a poxvirus.

**32**. A composition that comprises a bacterial vector comprising a DNA molecule of claim 3, said DNA molecule

being placed under conditions for expression and said bacterial vector being admixed with a physiologically acceptable diluent or carrier.

**33**. The composition of claim 32, wherein said vector is selected from the group consisting of Shigella, Salmonella, *Vibrio cholerae,* Lactobacillus, Bacille bilié de Calmette-Guérin, and Streptococcus.

**34**. A composition comprising a polynucleotide of claim 1, together with a physiologically acceptable diluent or carrier.

**35**. The composition of claim 34, wherein said polynucleotide is a DNA molecule that is inserted in a plasmid that is unable to replicate and to substantially integrate in a mammalian genome and is placed under conditions for expression in a mammalian cell.

**36**. An expression cassette comprising a DNA molecule of claim 3, said DNA molecule being placed under conditions for expression in a procaryotic or eucaryotic cell.

**37**. A process for producing a compound of claim 8, which comprises culturing a procaryotic or eucaryotic cell transformed or transfected with an expression cassette of claim 36, and recovering said compound from the cell culture.

**38**. A method of preventing or treating Helicobacter infection in a mammal, said method comprising administering to said mammal a prophylactically or therapeutically effective amount of an antibody that binds to the compound of claim 8.

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